

*JFW*

PATENT  
Client Reference Number: APB-2

**CERTIFICATE OF HAND DELIVERY**

I hereby certify that this correspondence is being hand filed with the United States Patent and Trademark Office in Washington, DC.  
20231 on NOV. 9. 2006.  
(Date)

Typed or printed name of person signing this certificate  
Dr. O. M. (Sam) Zaghmout

Signature Sam Zaghmout  
Attachments: European Patent application EP 02002250.5 (EP 1346998 A1).

**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

**Applicants:** Matthias Giese  
**Application Serial Number:** 10/675,444  
**Filing Date:** 09-30-2003  
**Title:** Equine Arteritis Virus Vaccine  
**Examiner:** Humphrey, Louise Wang Zhiying  
**Art Unit:** 1648  
**Confirmation Number:** 7837  
**Mail Stop:** Patent Application.

Date: November 7, 2006

Honorable Commissioner for Patents  
P.O. Box 1450  
Alexandria, VA 22313-1450

**TRANSMITTAL OF CERTIFIED COPY**

Sir:

Applicant in the above-entitled patent application hereby claims the date of priority of European Patent application EP 02002250.5 (EP 1346998 A1); title "Equine arteritis virus vaccine. The filing date of the European Patent application is January 30, 2002.

Attached please find the certified copy of the European Patent application from which priority is claimed for above captioned US patent application.

Applicant respectfully requests favorable consideration of the present application and a

**THIS PAGE BLANK (USPTO)**

timely examination of all of the pending claims.

Should any official at the United States Patent and Trademark Office deem that any further action by the Applicant or Applicant's undersigned representative is desirable and/or necessary, the official is invited to telephone the undersigned at the number set forth below.

The Commissioner is hereby authorized to charge any fees which may be required regarding this application under 37 CFR §§ 1.16-1.17 or credit any overpayment, to deposit account No. 503321. Should no proper payment be enclosed herewith, as by a check being in the wrong amount, unsigned, post-dated, or otherwise improper or informal or even entirely missing, the Commissioner is authorized to charge the unpaid amount to Deposit Account No. 503321.

Respectfully submitted,

By: Sam Zaghmout

O. M. (Sam) Zaghmout Ph.D  
(Registration No. 51,286)

**Contact Information:**

Bio Intellectual Property Service (BIO IPS) LLC  
8509 Kernon Ct, Lorton, VA 22079. USA  
Cell Phone (703-919-4348), Fax: (703-550-0409), (703) 550-1968 (Voice/Fax)

**THIS PAGE BLANK (USPTO)**





**Europäisches  
Patentamt**

**European  
Patent Office**

**Office européen  
des brevets**

**Bescheinigung**

**Certificate**

**Attestation**

Die angehefteten Unterla-  
gen stimmen mit der  
ursprünglich eingereichten  
Fassung der auf dem näch-  
sten Blatt bezeichneten  
europäischen Patentanmel-  
dung überein.

The attached documents  
are exact copies of the  
European patent application  
described on the following  
page, as originally filed.

Les documents fixés à  
cette attestation sont  
conformes à la version  
initialement déposée de  
la demande de brevet  
européen spécifiée à la  
page suivante.

**Patentanmeldung Nr.    Patent application No.    Demande de brevet n°**

02002250.5

Der Präsident des Europäischen Patentamts;  
Im Auftrag

For the President of the European Patent Office

Le Président de l'Office européen des brevets  
p.o.

**R C van Dijk**

**THIS PAGE BLANK (USPTO)**



Anmeldung Nr:  
Application no.: 02002250.5  
Demande no:

Anmeldetag:  
Date of filing: 30.01.02  
Date de dépôt:

Anmelder/Applicant(s)/Demandeur(s):

BOEHRINGER INGELHEIM VETMEDICA GMBH

55216 Ingelheim am Rhein  
ALLEMAGNE

Bezeichnung der Erfindung/Title of the invention/Titre de l'invention:  
(Falls die Bezeichnung der Erfindung nicht angegeben ist, siehe Beschreibung.  
If no title is shown please refer to the description.  
Si aucun titre n'est indiqué se referer à la description.)

Equine arteritis virus vaccine

In Anspruch genommene Priorität(en) / Priority(ies) claimed /Priorité(s)  
revendiquée(s)  
Staat/Tag/Aktenzeichen/State/Date/File no./Pays/Date/Numéro de dépôt:

Internationale Patentklassifikation/International Patent Classification/  
Classification internationale des brevets:

A61K39/00

Am Anmeldetag benannte Vertragstaaten/Contracting states designated at date of  
filing/Etats contractants désignées lors du dépôt:

AT BE CH CY DE DK ES FI FR GB GR IE IT LI LU MC NL PT SE TR

**THIS PAGE BLANK (USPTO)**

## Equine Arteritis Virus Vaccine

### Field of the invention

The invention belongs to the field of animal health and in equine arteritis virus (EAV). The invention provides vaccine compositions comprising open reading frame (ORF) 2, ORF 5 and ORF 7 nucleic acid of EAV, nucleic acid said ORF2, ORF 5 and ORF 7 and vectors comprising said ORFs. The invention further relates to the use of said ORFs and vectors in the manufacture of a medicament for the prevention and treatment of EAV infections.

### Background of the invention

Equine arteritis is a contagious disease of horses and is spread via respiratory or reproductive tract and caused by equine arteritis virus (EAV) which is a member of the *Arteriviridae* family, that includes lactate dehydrogenase-elevating virus (LDV), porcine reproductive and respiratory syndrome virus (PRRSV), and simian haemorrhagic fever virus (SHFV).

EAV is well investigated and its biological and biophysical properties together with the data on viral pathogenesis and cell virus interactions had been documented in over 200 scientific reports (enclosure 1). The genomic organization and transcriptional strategy of arteriviruses are shown in Fig. 1. The EAV virions are 60-65 nm in diameter and possess a 49S RNA genome that is a single-stranded, nonsegmented, capped and polyadenylated message-sense RNA (12687 nucleotides; den Boon, *et al.* 1991; GenBank accession number: X53459). The EAV genome is infectious and contains at least eight open reading frames (ORF) 1a, 1b, 2, 3, 4, 5, 6, and 7 (SEQ ID No. 10). The two largest viral ORFs (ORF 1a and ORF 1b) encode the viral replicase (den Boon, *et al.* 1991) and are located at the 5'-end of the viral genome between the nucleotide positions 1 and 9807. The ORFs 2 to 7 are overlapping and located at the 3'-end of EAV genome. These ORFs encode four known structural viral proteins and two proteins of unknown function (gene products of ORFs 3 and 4). The EAV virion consists of a phosphorylated nucleocapsid protein (N, 14 kDa, gene product of ORF 7), a N-glycosylated major membrane protein (G<sub>L</sub>, 30-44 kDa, gene product of ORF 5), a N-glycosylated minor membrane protein (G<sub>S</sub>, 25 kDa, gene product of ORF 2), and an unglycosylated membrane protein (M, 17 kDa, gene product of ORF 6).

The EAV infection is maintained in horse population, because chronic carrier animals shed EAV in their semen and transmit it venereally to mares during the mating season. PRRSV is also

characterized by reproductive failure e.g. late-term abortions in sows and causing respiratory illness and mortality in young pigs.

The analysis of the genetic stability of EAV during horizontal and vertical transmission in an outbreak of equine viral arteritis revealed that the carrier stallion is the source of genetic diversity of EAV (Balasuriya *et al.*, 1999). It is known that the infected carrier stallion is the critical natural reservoir of EAV. The outbreak of an EAV infection can be initiated by the horizontal aerosol transmission of specific viral variants that occur in the semen of carrier stallions. However, Patton and co-workers show that not only the carrier stallion is the critical natural reservoir of EAV, but also genetic diversity of the virus is generated in the course of persistent infection of carrier stallions (Patton *et al.*, 1999).

Consequently, PRRSV and EAV cause economically important infectious diseases in swine and horse farms worldwide. The development of an efficient vaccine is of particular importance, since it focuses attention on the prevention of the diseases.

In the art, there was a long lasting need for an effective EAV vaccine capable of preventing or curing an EAV-associated disease. Therefore, the technical problem underlying this invention was to provide such a vaccine capable of preventing or curing an EAV-associated disease.

## Figures

Fig. 1

Schematic diagram of the genomic organisation and transcriptional strategy of the family Arteriviridae.

Fig. 2:

Schematic diagram of the strategy used for molecular cloning of neutralizing domain of equine arteritis virus (EAV).

Fig. 3:

The results of neutralization tests obtained by the analysis of the sera of the individual Balb/c mice that were inoculated in two independent experiments (A and B) with the DNA of recombinant plasmid pCR3.1-EAV-O5-BX-C14 harboring and expressing ORF 5 of equine arteritis virus (EAV).

**Fig. 4:**

The results of neutralization test obtained by the analysis of the sera of the individual Balb/c mice that were inoculated with the DNA of recombinant plasmids pCR3.1-EAV-O5-BX-C14 and pCR3.1-EAV-O7-BX-C3 harboring and expressing ORFs 5 and 7 of equine arteritis virus (EAV).

**Fig. 5:**

The results of neutralization test obtained by the analysis of the sera of the individual Balb/c mice that were inoculated with the DNA of recombinant plasmids pDP-EAV-O5-BsS-C2 and pDP-EAV-O7-BsS-C1 harboring and expressing ORFs 5 and 7 of equine arteritis virus (EAV).

**Fig. 6:**

The results of neutralization test obtained by the analysis of the sera of the individual Balb/c mice that were inoculated with the DNA of recombinant plasmids pCR3.1-EAV-O5-BX-C14 and pCR3.1-EAV-O6-BE-C4 harboring and expressing ORFs 5 and 6 of equine arteritis virus (EAV).

**Fig. 7:**

The results of neutralization test obtained by the analysis of the sera of the individual Balb/c mice that were inoculated with the DNA of recombinant plasmid pCR3.1-EAV-O4-BX-C3 harboring and expressing ORF 4 of equine arteritis virus (EAV). The individual animals are indicated with number 1 to 10.

**Fig. 8:**

The results of neutralization test obtained by the analysis of the sera of the individual Balb/c mice that were inoculated with the DNA of recombinant plasmid pCR3.1-EAV-O4-BE-C3 harboring and expressing ORF 4 of equine arteritis virus (EAV). The individual animals are indicated with number 1 to 10.

**Fig. 9:**

The results of neutralization tests obtained by the analysis of the sera of the individual Balb/c mice that were inoculated with the DNA of recombinant plasmid pCR31-EAV-O5-del-121 harboring and expressing the N-terminal hydrophilic ectodomain of G<sub>L</sub> envelope glycoprotein (amino acid residue 1-121 of ORF 5) of equine arteritis virus (EAV).

**Fig. 10:**

The results of neutralization test obtained by the analysis of the sera of the individual Balb/c mice that were inoculated with the DNA of recombinant plasmids pCR3.1-EAV-O2-BX-C5, pCR3.1-EAV-O5-BX-C14, and pCR3.1-EAV-O6-BE-C4 harboring and expressing ORFs 2 (small glycoprotein), 5 (large envelope glycoprotein), and 6 (membrane protein), of equine arteritis virus (EAV).

**Fig. 11:**

The results of neutralization test obtained by the analysis of the sera of the individual Balb/c mice that were inoculated with the DNA of recombinant plasmids pCR3.1-EAV-O2-BX-C5 and pCR3.1-EAV-O4-BX-C3 harboring and expressing ORFs 2 and 4 of equine arteritis virus (EAV).

**Fig. 12:**

An example of the results obtained by enzyme linked immunosorbent assay (ELISA) for the detection of EAV specific antibodies.

**Fig. 13:** Establishing a lymphoproliferation assay for detection of cellular immune response

**Fig. 14 (Fig 14-14b)** Determination of the maximum cellular  $^{51}\text{Cr}$ -uptake

**Fig. 15 (Fig. 15a-15e)** Calculated specific lysis after the 2<sup>nd</sup> and 3<sup>rd</sup> booster immunization

**Disclosure of the invention****Definitions of terms used in the description:**

Before the embodiments of the present invention it must be noted that as used herein and in the appended claims, the singular forms "a", "an", and "the" include plural reference unless the context clearly dictates otherwise. Thus, for example, reference to "a nucleic acid molecule" includes a plurality of such nucleic acid molecules, reference to the "vector" is a reference to one or more vectors and equivalents thereof known to those skilled in the art, and so forth. Unless defined otherwise, all technical and scientific terms used herein have the same meanings as commonly understood by one of ordinary skill in the art to which this invention belongs.



Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods, devices, and materials are now described. All publications mentioned herein are incorporated herein by reference for the purpose of describing and disclosing the cell lines, vectors, and methodologies as reported in the publications which might be used in connection with the invention. Nothing herein is to be construed as an admission that the invention is not entitled to antedate such disclosure by virtue of prior invention.

The term "BAV" as used herein refers to all viruses belonging to species equine arteritis virus within the family Arteriviridae.

A „fragment“ according to the invention is any subunit of a DNA molecule (e.g. part of an open reading frame (ORF)) of a longer DNA molecule (e.g. an entire ORF) BAV according to the invention, i.e. any subset, characterized in that it is encoded by a shorter nucleic acid molecule than disclosed which can still be transcribed into RNA. „Fragment“ also relates to subsets of proteins, i.e. smaller proteins encoded by said DNA fragments. The expression is to be understood depending upon the context in which it is used.

A „functional variant“ of the DNA molecule according to the invention or protein encoded thereby is a DNA molecule or protein which possesses a biological activity (either functional or structural) that is substantially similar to the DNA molecule or protein according to the invention. The term „functional variant“ also includes „a fragment“, „a functional variant“, „variant based on the degenerative nucleic acid code“ or „chemical derivative“. Such a „functional variant“ e.g. may carry one or several nucleic acid exchanges, deletions or insertions. Said exchanges, deletions or insertions may account for 10% of the entire sequence. Said functional variant at least partially retains its biological activity, e.g. function as an infectious clone or a vaccine strain, or even exhibits improved biological activity.

A „variant based on the degenerative nature of the genetic code“ is a variant resulting from the fact that a certain amino acid may be encoded by several different nucleotide triplets. Said variant at least partially retains its biological activity, or even exhibits improved biological activity.

According to the invention, "mutation" means the replacement of a nucleotide by another (e.g. C for a T) a so-called "substitution" or any other mutation such as "deletion" or "insertion". "Deletion" means the removal of one or several nucleotides or amino acids.

A „fusion molecule“ may be the DNA molecule or protein according to the invention fused to e.g. a reporter such as a radiolabel, a chemical molecule such as a fluorescent label or any other molecule known in the art.

As used herein, a „chemical derivative“ according to the invention is a DNA molecule or protein according to the invention chemically modified or containing additional chemical moieties not normally being part of the molecule. Such moieties may improve the molecule's solubility, absorption, biological half life etc.

A molecule is „substantially similar“ to another molecule if both molecules have substantially similar nucleotide sequences or biological activity. Thus, provided that two molecules possess a similar activity, they are considered variants as that term is used herein if the nucleotide sequence is not identical, and two molecules which have a similar nucleotide sequence are considered variants as that term is used herein even if their biological activity is not identical.

The terms „vaccine“ and „vaccine composition“ are used interchangeably.

The term „vaccine“ as used herein refers to a pharmaceutical composition comprising at least one immunologically active component that induces an immunological response in an animal and possibly but not necessarily one or more additional components that enhance the immunological activity of said active component. A vaccine may additionally comprise further components typical to pharmaceutical compositions. The immunologically active component of a vaccine may comprise complete virus particles in either their original form or as attenuated particles in a so called modified live vaccine (MLV) or particles inactivated by appropriate methods in a so called killed vaccine (KV). In another form the immunologically active component of a vaccine may comprise appropriate elements of said organisms (subunit vaccines) whereby these elements are generated either by destroying the whole particle or the growth cultures containing such particles and optionally subsequent purification steps yielding the desired structure(s), or by synthetic processes including an appropriate manipulation by use of a suitable system based on, for example, bacteria, insects, mammalian or other species plus optionally subsequent isolation and purification procedures, or by induction of said synthetic processes in the animal needing a vaccine by direct incorporation of genetic material using suitable pharmaceutical compositions (polynucleotide vaccination). A vaccine may comprise one or simultaneously more than one of the elements described above.

The term „vaccine“ as understood herein is a vaccine for veterinary use comprising antigenic substances and is administered for the purpose of inducing a specific and active immunity against a disease provoked by EAV. The EAV vaccine according to the invention confers active

immunity that may be transferred passively via maternal antibodies against the immunogens it contains and sometimes also against antigenically related organisms.

Additional components to enhance the immune response are constituents commonly referred to as adjuvants, like e.g. aluminiumhydroxide, mineral or other oils or ancillary molecules added to the vaccine or generated by the body after the respective induction by such additional components, like but not restricted to interferons, interleukins or growth factors.

A "vaccine composition" essentially consists of one or more ingredients capable of modifying physiological e.g. immunological functions of the organism it is administered to, or of organisms living in or on the organism. The term includes, but is not restricted to antibiotics or antiparasitics, as well as other constituents commonly used to achieve certain other objectives like, but not limited to, processing traits, sterility, stability, feasibility to administer the composition via enteral or parenteral routes such as oral, intranasal, intravenous, intramuscular, subcutaneous, intradermal or other suitable route, tolerance after administration, controlled release properties.

#### Disclosure of the invention

The solution to the above technical problem is achieved by the description and the embodiments characterized in the claims.

The long lasting need in the art for a vaccine capable of preventing or curing an EAV-associated disease has been overcome by the present inventors who provided a such a nucleic acid-based prophylactic or therapeutic vaccine for EAV-associated diseases. Said vaccine is described in more details *infra*.

Surprisingly, the nucleic acid-based vaccine according to the invention for the first time in the art is capable of not only generating a humoral (antibody-based) response (demonstrated in an exemplary manner in e.g. example 1), but also a cellular immune response in horses. Only this cellular immune response, as exemplified in examples 2 to 5, is protective against horizontal and vertical EAV transmission in horses. More surprisingly, quite contrary to what was expected by the artisan (Barry and Johnston, 1997) the vaccine according to the invention is protective against infection not only in young horses, but also in horses of all ages (as exemplified by data, see table 8 and 10). Thus, the vaccines according to the invention as described *infra* are capable of inducing a cellular immune response in horses and are protective against horizontal and vertical EAV transmission in horses of different ages.

In a first important embodiment, the invention relates to a vaccine composition which is protective against equine arterivirus (EAV) infections in horses and induces a cellular immune response, comprising a open reading frame nucleic acid (ORF) 2, ORF 5 and/or ORF7 of EAV.

The invention also relates to vaccine compositions wherein said ORF 2, ORF5, and/or ORF7 are fragments, functional variants or carry mutations as defined *supra*.

To prepare such nucleic acids, the artisan may follow the examples of the present invention and apply also standard molecular biology methods which can be found e.g. in Sambrook et al. (1989) *Molecular Cloning: A Laboratory Manual*, 2<sup>nd</sup> ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York and Bertram, S. and Gassen, H.G. *Gentechnische Methoden*, G. Fischer Verlag, Stuttgart, New York, 1991).

The invention further relates to a vaccine composition according to the invention, wherein said vaccine composition comprises a nucleic acid ORF 2, ORF 5 and ORF7 of EAV. Surprisingly, contrary to the opinion in the art, a vaccine comprising nucleic acid said three ORFs is particularly effective and much better than a vaccine wherein the entire cDNA for EAV is used. ORF 2 relates also to ORF 2a which is comprised by the present invention. ORF 2 encodes a small glycoprotein. ORF 5 encodes a large envelope glycoprotein. ORF 7 encodes the nucleocapsid protein. The invention also relates to vaccine compositions comprising mutated or truncated ORFs, such as ORF 5 in a deleted form (SEQ ID No. 9).

The invention further relates to a vaccine composition according to the invention as disclosed *supra*, wherein said vaccine composition further comprises one or several ORFs selected from the group of ORF 1a, ORF 1b, ORF 3, ORF 4, ORF 6. ORF 1a and ORF 1b encode the viral replicase. Any combination of said ORFs or all of said ORFs may be part of said vaccine. ORF 3 and ORF 4 encode proteins of yet unknown function. ORF 6 encodes an unglycosylated membrane protein. Preferably, said ORF or ORFs are as disclosed in SEQ ID No. 1 or SEQ ID No. 8, SEQ ID No. 3, SEQ ID No. 4, SEQ ID No. 6.

The invention also relates to vaccine compositions comprising mutated or truncated ORFs, such as the partial ORF1 sequence in SEQ ID No. 8.

The invention further relates to a vaccine composition according to the invention as disclosed *supra*, wherein said nucleic acid is cDNA.

The invention further relates to a vaccine composition according to the invention as disclosed *supra*, wherein said vaccine composition comprises one or several nucleic acid vectors each comprising said ORF or ORFs. One embodiment of the invention relates to such a vector comprising more than one ORF.

The invention further relates to a vaccine composition according to the invention as disclosed *supra*, wherein said vector(s) is/are expression vector(s).

The invention further relates to a vaccine composition according to the invention as disclosed *supra*, wherein said expression vector(s) comprise(s) a eukaryotic cis-acting transcription/translation sequence functionally linked to said ORF(s).

The invention further relates to a vaccine composition according to the invention as disclosed *supra*, wherein said expression vector is selected from the group of pCR3.1, pcDNA3.1/His A, pcDNA3.1/His B, pcDNA3.1/His C, and pDisplay (pD) Such vectors are commercially available (Invitrogen).

The invention further relates to a vaccine composition according to the invention as disclosed *supra*, further comprising the nucleic acid encoding interleukin 2 (IL-2) or a vector or expression vector comprising said nucleic acid encoding IL-2.

The invention further relates to a vaccine composition according to the invention as disclosed *supra*, further comprising pharmaceutically acceptable carrier or excipient.

The invention further relates to a vaccine composition according to the invention as disclosed *supra*, further comprising one or several adjuvants selected from the group of Muramyl Dipeptide (MDP), Montanide 720, Poly Inosine:Cytosine (Poly I:C) or plasmid DNA comprising unmethylated cytosine, guanine dinucleotide sequence motifs (CpG).

The invention relates to a vaccine according to the invention as described *supra* wherein the adjuvants is any one of the compounds described in Chapter 7 (pp 141-227) of "Vaccine Design, The Subunit and Adjuvant Approach" (eds. Powell, M. F. and Newman, M. J.) Pharmaceutical Biotechnology, Volume 6, Plenum Press (New York). Examples from this compendium include Muramyl Dipeptide (MDP) and Montanide 720 as disclosed *supra*. Molecules such as Poly

Inosine:Cytosine (Poly I:C) or „immunostimulatory nucleic acid molecules“ such as plasmid DNA containing CpG motifs can also be administered as adjuvants in combination with antigens encapsulated in microparticles. An "immunostimulatory nucleic acid molecule" refers to a nucleic acid molecule, which contains an unmethylated cytosine, guanine dinucleotide sequence (i.e. "CpG DNA" or DNA containing a cytosine followed by guanosine and linked by a phosphate bond) and stimulates (e.g. has a mitogenic effect on, or induces or increases cytokine expression by) a vertebrate lymphocyte. An immunostimulatory nucleic acid molecule can be double-stranded or single-stranded. Generally, double-stranded molecules are more stable *in vivo*, while single-stranded molecules have increased immune activity. The instant invention is based on the finding that certain "immunostimulatory nucleic acid molecules" containing unmethylated cytosine-guanine (CpG) dinucleotides activate lymphocytes in a subject and redirect a subject's immune response from a Th2 to a Th1 (e.g. by inducing monocytic cells and other cells to produce Th1 cytokines, including IL-12, IFN- $\gamma$ , and GM-CSF).

The invention further relates to a vaccine composition according to the invention as disclosed *supra*, consisting of expression vectors comprising ORF2, ORF5 and ORF7 of EAV, respectively, and optionally carrier, excipients or adjuvants and an expression vector comprising the nucleic acid encoding IL-2. Said nucleic acid is preferably equine IL-2. Preferably also, said the coding nucleic acid equine IL-2 is co-expressed on a vector as disclosed *supra* encoding one or several EAV ORFs.

The invention further relates to a vaccine composition according to the invention as disclosed *supra*, wherein ORF 2 is SEQ ID No. 2, ORF 5 is SEQ ID No. 5 or SEQ ID No. 9 and ORF 7 is SEQ ID No. 7.

Suitable for targeted delivery of the vaccine composition according to the invention are colloidal dispersion systems or liposomes. One example of a targeted delivery system for the EAV ORF nucleic acid molecules according to the invention is said colloidal dispersion system. Colloidal dispersion systems include macromolecule complexes, nanocapsules, microspheres, beads, and lipid-based systems including oil-in-water emulsions, micelles, mixed micelles, and liposomes or liposome formulations. The preferred colloidal system of this invention is a liposome. Liposomes are artificial membrane vesicles which are useful as delivery vehicles *in vitro* and *in vivo*. These formulations may have net cationic, anionic or neutral charge characteristics are useful characteristics with *in vitro*, *in vivo* and *ex vivo* delivery methods. It has been shown that large

unilamellar vesicles (LUV), which range in size from 0.2-4.0  $\mu\text{m}$  can encapsulate a substantial percentage of an aqueous buffer containing large macromolecules. RNA, DNA and intact virions can be encapsulated within the aqueous interior and be delivered to cells in a biologically active form (Fraley R and Papahadjopoulos D (1981). New generation liposomes - The engineering of an efficient vehicle for intracellular delivery of nucleic acids. Trends Biochem Sci 6, 77-80). In addition to mammalian cells, liposomes have been used for delivery of polynucleotides in plant, yeast and bacterial cells. In order for a liposome to be an efficient gene transfer vehicle of the EAV ORFs according to the invention, the following characteristics should be present: (1) encapsulation of the genes of interest at high efficiency while not compromising their biological activity; (2) preferential and substantial binding to a target cell in comparison to non-target cells; (3) delivery of the aqueous contents of the vesicle to the target cell cytoplasm at high efficiency; and (4) accurate and effective expression of genetic information (Mannino RJ, and Gould-Fogerite S (1988). Liposome mediated gene transfer. BioTechniques 6, 682-690.).

The composition of the liposome is usually a combination of phospholipids, particularly high-phase-transition-temperature phospholipids, usually in combination with steroids, especially cholesterol. Other phospholipids or other lipids may also be used. The physical characteristics of liposomes depend on pH, ionic strength, and the presence of divalent cations.

The vaccine composition of the present invention may contain said recombinant vector as a naked "gene expression vector". This means that the construct is not associated with a delivery vehicle (e.g. liposomes, colloidal particles and the like). One of the principal advantages of naked DNA vectors is the lack of an immune response stimulated by the vector itself.

The invention further relates to a vaccine composition according to the invention as disclosed *supra*, wherein the nucleic acid or nucleic acid vector or expression vector is encapsulated into liposomes.

Several types of liposomal preparations may be used for encapsulation, including large multilamellar vesicles, small unilamellar vesicles, neutral, anionic liposomes or simple cationic amphiphiles. Most preferred are cationic liposomes.

These synthetic gene delivery systems are described by many terms:

The cationic lipid-mediated transfection has been also called liposome-mediated-transfection, cationic liposome-mediated transfection, lipofection, cytofection, amphifection, and lipid-mediated transfection. Similarly, the complexes that are produced when cationic lipids are

mixed with DNA have been referred to as cytosomes, amphisomes, liposomes, nucleolipidic particles, cationic lipid-DNA complexes, lipid-DNA complexes, DNA-lipid complexes etc. Recently, a common nomenclature was proposed: *Lipoplex* - replaces all of the terms for cationic lipid-nucleic acid complexes (including DNA, RNA, or synthetic oligonucleotides) and *lipofection* means the nucleic acid delivery mediated by lipoplexes. Any of said gene delivery system may be used according to the invention.

The positive charge on cationic lipid molecules facilitates their association with negatively charged nucleic acid as well as with membrane phospholipids (negatively charged) what is the basis for the non-specific interaction of the complex.

The specific binding to the cell is mediated by use of specific ligands for cellular receptors. Cationic Liposomes may deliver DNA either directly across the plasma membrane or via endosome compartment. Regardless of its exact entry point, much of the DNA does accumulate in the endosomes and is lost by the internal hydrolytic digestion within the endosomes. To protect the plasmid DNA several strategies may be used according to the invention. This includes the use of acidotropic, weak amines such as chloroquine, which presumably prevent DNA degradation by inhibiting endosomal acidification. But also viral fusion peptides or whole viruses may be included to disrupt endosomes or promote fusion of liposomes with endosomes and facilitate release of DNA into the cytoplasm. Such protection of the plasmid DNA is also a preferred embodiment of the invention.

The DNA concentration, the ratio of lipid reagent to DNA, the transfection time and the effect of serum are the most critical factors in each transfection.

Liposomes must be stable. In case of leakage they would lose antigen and adjuvants premature.

Preferred is a vaccine composition according to the invention as disclosed *supra* comprising 0.05 µg - 10 µg, preferred 0.1 µg - 1 µg, most preferred 0.5 µg. Further preferred dose ranges are: low range 0.1-1.0 µg, most preferred 0.5 µg, middle range 1.1-10 µg, most preferred 15 µg, high range 11 µg-20 µg, most preferred 15 µg. The artisan knows the criteria for the ideal dose which depends on the chosen route of administration, i.e. with gene gun the vaccine is injected directly into Langerhans cells, thus very little of antigen gets lost, whereas i.m. injection requires much higher doses.

Most preferred is a vaccine composition according to the invention as disclosed *supra* comprising 0.5 µg of individual nucleic acid vector or preferred expression vector and preferably for 10 shots per animal, i.e. 5 µg of individual nucleic acid vector (or preferred expression vector)



per vaccination, e.g. if seven nucleic acid vectors (or preferred expression vectors) are used, 35 µg per vaccination and animal (see example 2).

Also preferred is a vaccine composition according to the invention as disclosed *supra* comprising: low range 10-100 µg, most preferred 50 µg, middle range 101-500 µg, most preferred 200 µg, high range 501 µg-2000 µg, most preferred 1000 µg. Again, the artisan knows the criteria for the ideal dose which depends on the chosen route of administration, i.e. with gene gun the vaccine is injected directly into Langerhans cells, thus very little of antigen gets lost, whereas i.m. injection requires much higher doses.

Also most preferred is a vaccine composition according to the invention as disclosed *supra* comprising 50 µg of individual nucleic acid vector or preferred expression vector and preferably for 4 injections per animal, i.e. 200 µg of individual nucleic acid vector (or preferred expression vector) per vaccination, e.g. if seven nucleic acid vectors (or preferred expression vectors) are used, 1,4 mg per vaccination and animal (see example 2).

In yet another important embodiment the invention relates to a nucleic acid vector comprising nucleic acid selected from the group of ORF 1a, ORF 1b, ORF 2, ORF 3, ORF 4, ORF 5, ORF 6 and/or ORF7 of EAV. Preferably, said ORF or ORFs are as disclosed in SEQ ID No. 1 or SEQ ID No. 8, SEQ ID No. 2, SEQ ID No. 3, SEQ ID No. 4, SEQ ID No. 5, SEQ ID No. 6 and/or SEQ ID No. 7.

A preferred aspect of the invention is a nucleic acid vector according to the invention as disclosed *supra*, wherein said nucleic acid is DNA.

A more preferred aspect of the invention is a nucleic acid vector according to the invention as disclosed *supra*, wherein said nucleic acid vector is an expression vector.

Another more preferred aspect of the invention is a nucleic acid vector according to the invention as disclosed *supra*, wherein said expression vector comprises a eukaryotic cis-acting transcription/translation sequence functionally linked to said ORF(s).

To accomplish expression, a wide variety of vectors have been developed and are commercially available which allow inducible (e.g., LacSwitch expression vectors, Stratagene, La Jolla, CA) or cognate (e.g., pcDNA3 vectors, Invitrogen, Chatsworth, CA) expression of EAV ORF nucleotide

sequences under the regulation of an artificial promoter element. Such promoter elements are often derived from CMV or SV40 viral genes, although other strong promoter elements which are active in eukaryotic cells can also be employed to induce transcription of EAV ORF nucleotide sequences. Typically, these vectors also contain an artificial polyadenylation sequence and 3' UTR which can also be derived from exogenous viral gene sequences or from other eukaryotic genes. Furthermore, in some constructs, artificial, non-coding, spliceable introns and exons are included in the vector to enhance expression of the nucleotide sequence of interest (in this case, EAV ORF sequences). These expression systems are commonly available from commercial sources and are typified by vectors such as pCDNA3 and pZeoSV (Invitrogen, San Diego, CA). Innumerable commercially-available as well as custom-designed expression vectors are available from commercial sources to allow expression of any desired EAV ORF transcript in more or less any desired cell type, either constitutively or after exposure to a certain exogenous stimulus (e.g., withdrawal of tetracycline or exposure to IPTG).

A most preferred aspect of the invention is a nucleic acid according to the invention as disclosed *supra*, wherein said expression vector is selected from the group of pCR3.1, pcDNA3.1/His A, pcDNA3.1/His B, pcDNA3.1/His C, and pDisplay (pD).

Another most preferred aspect of the invention is a nucleic acid according to the invention as disclosed *supra*, wherein said nucleic acid vector comprises a nucleic acid selected from the group of SEQ ID No. 2, SEQ ID No. 5, SEQ ID No. 9 and/or SEQ ID No. 7.

Gene gun is a ballistic system that can propel DNA-coated microparticles directly into the skin. Plasmid DNA is affixed to gold particles of about 0,45  $\mu\text{m}$ . The total amount of DNA per sheet is a function of the DNA/gold ratio, i.e. 5  $\mu\text{g}$  DNA/mg gold. If about 0,4 mg of gold particles are shoot into the epidermis/dermis driven by helium (helium discharge pressure of 400-450 psi), about 2  $\mu\text{g}$  of DNA is inoculated into the skin.

Therefore, the fundamental difference of gene gun vs. i.m. injection is the amount of DNA required to produce an equivalent level of gene expression. This apparent difference could most likely related to the fact that i.m. or i.d. injections by needle places the DNA also into extracellular spaces, exposure of DNA to nucleases in the interstitial fluid or that the hydrostatic pressure that results from the injection of several  $\mu\text{l}$  of saline into a muscle rapidly drives out the DNA of protein producing cells so that only a little amount of injected DNA will be expressed in protein.

Interestingly, the gun is in general limited by the little amount of plasmid that can be delivered in one inoculation. This limit is about 2,5 µg of plasmid per shot. Exceeding this amount causes the particles to clump together, creating 'macroparticles' which cause increased damage to the target tissue. By contrast, milligrams of plasmid DNA can be delivered by i.m. injections using needles and the success of i.m. injection can be easily monitored by swelling of the injected muscle bundle. Qualitative differences in immunisation by the gene gun and i.m. injection can be circumvented by increasing the amount of DNA. A DNA inoculation by gene gun needs almost hairless skin. So the most animals to be vaccinated have to get shaved.

Such problems should be overcome by new generations of gene guns which also may be used according to the invention. A new device, the PowderJect<sup>®</sup> system driven by helium gas, is the first one which is able to deliver both metallic and non-metallic particles into tissues. Most preferred is the use of the PowderJect<sup>®</sup> system.

Thus, another important embodiment of the invention is a method for prophylaxis or treatment of EAV infection in a horse, comprising

(i) coating one or several DNA molecule(s) according to the invention as disclosed *supra*, or one or several nucleic acid vector(s) according to the invention as disclosed *supra*, onto carrier particles;

(ii) accelerating the coated carrier particles into epidermal cells of the horse *in vivo*; and

(iii) inducing a protective or therapeutic immune response in said horse upon or after exposure to EAV; and

(iv) monitoring the reduction of EAV-associated symptoms or the reduction of horizontal or vertical transmission.

Preferably, in said method as disclosed *supra*, the carrier particles are gold.

Any of the above-disclosed devices such as gene gun or the PowderJect<sup>®</sup> system may be used. The injection may be carried out as disclosed *supra*. Most preferably, said method may be carried out repeatedly. An appropriate vaccination scheme may be preferably on day 0 (basic vaccination), 2 weeks thereafter, 4 weeks after the basic vaccination and 7 weeks after the basic vaccination. This is exemplified in example 2.

Also preferred vaccination schemes are:

- 1) only one base immunization without booster; or
- 2) base immunization, 1<sup>st</sup> boost after 8-12 weeks; or
- 3) base immunization, 1<sup>st</sup> boost after 8-12 weeks, 2<sup>nd</sup> boost after 12 months.

Said vaccine, DNA molecule(s) according to the invention as disclosed *supra*, or one or several nucleic acid vector(s) according to the invention as disclosed *supra* may be administered by any known route of administration: preferably orally, nasally, lingually, intravenously (i.v.), intradermally (i.d.), intraepidermally (by rubbing into the skin), intranasally, vaginally, subcutaneously (s.c.), intramuscular (i.m.).

According to the invention, various vehicles for administration of the vaccine, DNA molecule(s) according to the invention as disclosed *supra*, or one or several nucleic acid vector(s) according to the invention as disclosed *supra* may be used: only plasmid 'naked' DNA inoculation by needle - liposomes - gold beads - biodegradable nanoparticles - virus like particles (VLP) - aerosol.

Preferred modes of administration for the vaccine, DNA molecule(s) according to the invention as disclosed *supra*, or one or several nucleic acid vector(s) according to the invention as disclosed *supra* are: injection by needle, gene gun, encapsulated in liposomes or rubbing into the skin.

Preferred doses are 0.5 µg of individual nucleic acid vector or preferred expression vector and 10 shots per animal, i.e. 5 µg of individual nucleic acid vector (or preferred expression vector) per vaccination, e.g. if seven nucleic acid vectors (or preferred expression vectors) are used, 35 µg per vaccination and animal (see example 2).

Preferred are 1 to 10, more preferably 5 or 7 or 10 shots per animal, most preferred are 10 shots per animal.

One preferred method of vaccination is the direct injection of plasmid DNA into skeletal muscle. Long-lasting immune responses are obtained in many cases without boost.

For the i.m. route, DNA preferably is injected by needle, whereas for the i.e. route, a gene gun preferably is used (see *supra*):

Yet another important embodiment of the invention is a method for prophylaxis or treatment of EAV infection in a horse, comprising

- (i) injecting a vaccine composition according to the invention as disclosed *supra*, or one or several DNA molecule(s) according to the invention as disclosed *supra*, or one or several nucleic acid vector(s) according to the invention as disclosed *supra* into muscular cells of the horse *in vivo*; and
- (ii) inducing a protective or therapeutic immune response in said horse upon or after exposure to EAV, and
- 10 (iii) monitoring the reduction of EAV-associated symptoms or the reduction of horizontal or vertical transmission.

Most preferably, said method may be carried out repeatedly. An appropriate vaccination scheme may be preferably on day 0 (basic vaccination), 2 weeks thereafter, 4 weeks after the basic  
15 vaccination and 7 weeks after the basic vaccination. This is exemplified in example 2. Preferred doses are 50 µg of individual nucleic acid vector or preferred expression vector, and 4 inoculations per animal, i.e. 200 µg of individual nucleic acid vector (or preferred expression vector) per vaccination, e.g. if seven nucleic acid vectors (or preferred expression vectors) are used, 1,4 mg per vaccination and animal (see example 2).

Also preferred is a vaccine composition according to the invention as disclosed *supra* comprising: low range 10-100 µg, most preferred 50 µg, middle range 101-500 µg, most preferred 200 µg, high range 501µg-2000µg, most preferred 1000µg. Again, the artisan knows  
20 the criteria for the ideal dose which depends on the chosen route of administration, i.e. with gene gun the vaccine is injected directly into Langerhans cells, thus very little of antigen gets lost, whereas i.m. injection requires much higher doses.

Again, preferred vaccination schemes are:

- 1) only one base immunization without booster; or
- 20 2) base immunization, 1<sup>st</sup> boost after 8-12 weeks; or
- 3) base immunization, 1<sup>st</sup> boost after 8-12 weeks, 2<sup>nd</sup> boost after 12 months.

Said vaccine, DNA molecule(s) according to the invention as disclosed *supra*, or one or several nucleic acid vector(s) according to the invention as disclosed *supra* may be administered by any

known route of administration: preferably orally, intravenously (i.v.), intradermally, intraepidermally (by rubbing into the skin), intranasally, vaginally, subcutaneously (s.c.), intramuscular (i.m.).

Preferred modes of administration for the vaccine, DNA molecule(s) according to the invention as disclosed *supra*, or one or several nucleic acid vector(s) according to the invention as disclosed *supra* are: injection by needle, gene gun, encapsulated in liposomes or rubbing into the skin.

Preferred are 1 to 10, more preferably 3 or 4 or 5 inoculations per animal, most preferred are 4 inoculations per animal.

The invention further relates to the use of one or several DNA molecule(s) according to the invention as disclosed *supra* or one or several nucleic acid vector(s) according to the invention as disclosed *supra* in the manufacture of a vaccine for the prophylaxis and treatment of EAV infections. Preferred is the use of DNA molecule(s) or nucleic acid vector(s) according to the invention as disclosed *supra* in the manufacture of a vaccine suitable for intraepidermal, intradermal or intramuscular administration for the prophylaxis and treatment of EAV infections.

More preferred is the use of DNA molecule(s) or nucleic acid vector(s) according to the invention as disclosed *supra* in the manufacture of a vaccine suitable for intraepidermal or intradermal administration for the prophylaxis and treatment of EAV infections, wherein the vaccine is to be administered on day 0 (basic vaccination), 2 weeks thereafter, 4 weeks after the basic vaccination and 7 weeks after the basic vaccination. This is exemplified in example 2.

More preferred is the use of DNA molecule(s) or nucleic acid vector(s) or expression vector according to the invention as disclosed *supra* in the manufacture of a vaccine suitable for intraepidermal or intradermal administration for the prophylaxis and treatment of EAV infections, wherein the vaccine comprises 0.5 µg of individual nucleic acid vector or preferred expression vector and preferably for 10 shots per animal, i.e. 5 µg of individual nucleic acid vector (or preferred expression vector) per vaccination, e.g. if seven nucleic acid vectors (or preferred expression vectors) are used, 35 µg per vaccination and animal (see example 2).

Preferred is the use of DNA molecule(s) or nucleic acid vector(s) or expression vector according to the invention comprising 0.05  $\mu\text{g}$  – 10  $\mu\text{g}$ , preferred 0.1  $\mu\text{g}$  – 1  $\mu\text{g}$ , most preferred 0.5  $\mu\text{g}$ . Further preferred dose ranges are: low range 0.1-1.0  $\mu\text{g}$ , most preferred 0.5  $\mu\text{g}$ , middle range 1.1-10  $\mu\text{g}$ , most preferred 15  $\mu\text{g}$ , high range 11  $\mu\text{g}$ -20  $\mu\text{g}$ , most preferred 15  $\mu\text{g}$ . The artisan knows the criteria for the ideal dose which depends on the chosen route of administration, i.e. with gene gun the vaccine is injected directly into Langerhans cells, thus very little of antigen gets lost, whereas i.m. injection requires much higher doses.

Again, preferred vaccination schemes are:

- 1) only one base immunization without booster; or
- 2) base immunization, 1<sup>st</sup> boost after 8-12 weeks; or
- 3) base immunization, 1<sup>st</sup> boost after 8-12 weeks, 2<sup>nd</sup> boost after 12 months.

Said vaccine, DNA molecule(s) according to the invention as disclosed *supra*, or one or several nucleic acid vector(s) according to the invention as disclosed *supra* may be administered by any known route of administration: preferably orally, intravenously (i.v.), intradermally, intraepidermally (by rubbing into the skin), intranasally, vaginally, subcutaneously (s.c.), intramuscular (i.m.).

Preferred modes of administration for the vaccine, DNA molecule(s) according to the invention as disclosed *supra*, or one or several nucleic acid vector(s) according to the invention as disclosed *supra* are: injection by needle, gene gun, encapsulated in liposomes or rubbing into the skin.

Preferred are 1 to 10, more preferably 3 or 4 or 5 administrations per animal, most preferred are 4 administrations per animal.

More preferred is the use of DNA molecule(s) or nucleic acid vector(s) according to the invention as disclosed *supra* in the manufacture of a vaccine suitable for intraepidermal or intradermal administration for the prophylaxis and treatment of EAV infections, wherein the vaccine is to be administered on day 0 (basic vaccination), 2 weeks thereafter, 4 weeks after the basic vaccination and 7 weeks after the basic vaccination. This is exemplified in example 2.

Preferred is the use of DNA molecule(s) or nucleic acid vector(s) or expression vector according to the invention comprising 0.05  $\mu$ g - 10  $\mu$ g, preferred 0.1  $\mu$ g - 1  $\mu$ g, most preferred 0.5  $\mu$ g. Further preferred dose ranges are: low range 0.1-1.0  $\mu$ g, most preferred 0.5  $\mu$ g, middle range 1.1-10  $\mu$ g, most preferred 15  $\mu$ g, high range 11  $\mu$ g-20  $\mu$ g, most preferred 15  $\mu$ g. The artisan knows the criteria for the ideal dose which depends on the chosen route of administration.

Again, preferred vaccination schemes are:

- 1) only one base immunization without booster; or
- 2) base immunization, 1<sup>st</sup> boost after 8-12 weeks; or
- 3) base immunization, 1<sup>st</sup> boost after 8-12 weeks, 2<sup>nd</sup> boost after 12 months.

Said vaccine, DNA molecule(s) according to the invention as disclosed *supra*, or one or several nucleic acid vector(s) according to the invention as disclosed *supra* may be administered by any known route of administration: preferably orally, intravenously (i.v.), intradermally, intraepidermally (by rubbing into the skin), intranasally, vaginally, subcutaneously (s.c.), intramuscular (i.m.).

Preferred modes of administration for the vaccine, DNA molecule(s) according to the invention as disclosed *supra*, or one or several nucleic acid vector(s) according to the invention as disclosed *supra* are: injection by needle, gene gun, encapsulated in liposomes or rubbing into the skin.

Preferred are 1 to 10, more preferably 3 or 4 or 5 administrations per animal, most preferred are 4 administrations per animal.

Also more preferred is the use of DNA molecule(s) or nucleic acid vector(s) or expression vector according to the invention as disclosed *supra* in the manufacture of a vaccine suitable for intramuscular administration for the prophylaxis and treatment of EAV infections, wherein the vaccine comprises 50  $\mu$ g of individual nucleic acid vector or preferred expression vector and preferably for 4 shots per animal, i.e. 200  $\mu$ g of individual nucleic acid vector (or preferred expression vector) per vaccination, e.g. if seven nucleic acid vectors (or preferred expression vectors) are used, 1,4 mg per vaccination and animal (see example 2).



Also preferred is use of DNA molecule(s) or nucleic acid vector(s) or expression vector according to the invention as disclosed *supra* comprising: low range 10-100 µg, most preferred 50 µg, middle range 101-500 µg, most preferred 200 µg, high range 501µg-2000µg, most preferred 1000µg. Again, the artisan knows the criteria for the ideal dose which depends on the chosen route of administration, i.e. with gene gun the vaccine is injected directly into Langerhans cells, thus very little of antigen gets lost, whereas i.m. injection requires much higher doses.

Again, preferred vaccination schemes are:

- 1) only one base immunization without booster; or
- 2) base immunization, 1<sup>st</sup> boost after 8-12 weeks; or
- 3) base immunization, 1<sup>st</sup> boost after 8-12 weeks, 2<sup>nd</sup> boost after 12 months.

Said vaccine, DNA molecule(s) according to the invention as disclosed *supra*, or one or several nucleic acid vector(s) according to the invention as disclosed *supra* may be administered by any known route of administration: preferably orally, intravenously (i.v.), intradermally, intraepidermally (by rubbing into the skin), intranasally, vaginally, subcutaneously (s.c.), intramuscular (i.m.).

Preferred modes of administration for the vaccine, DNA molecule(s) according to the invention as disclosed *supra*, or one or several nucleic acid vector(s) according to the invention as disclosed *supra* are: injection by needle, gene gun, encapsulated in liposomes or rubbing into the skin.

Preferred are 1 to 10, more preferably 3 or 4 or 5 administrations per animal, most preferred are 4 administrations per animal.

The invention also relates to the a kit of parts comprising a vaccine composition according to the invention as disclosed *supra* or one or several EAV ORF DNA molecule(s) according to the invention as disclosed *supra* or one or several nucleic acid vector(s) according to the invention as disclosed *supra*. Said kit is ready-to-use for vaccination of horses. Said kit may further contain, but is not limited to test tubes, other suitable containers, washing reagents and reaction buffers (which may vary in pH and magnesium concentrations), sterile water, liposomal preparations, transfection reagent such as DOTAP Liposomal (Roche) or Lipofectin, BME (Eagle's basal

medium), ethanol, gold, spermidine,  $\text{CaCl}_2$ , carrier proteins and further compounds known to the skilled artisan.

The following examples serve to further illustrate the present invention; but the same should not be construed as limiting the scope of the invention disclosed herein.

### Example 1 Neutralization tests

## MATERIALS AND METHODS

### Viruses and cells

The equine arteritis virus (EAV) used in this study was kindly provided by Professor H. Ludwig, Berlin and propagated on rabbit kidney cells (RK13, ATCC number CCL-37). The cell cultures were obtained from the American Type Culture Collection and propagated in Basal Medium Eagle (BME) supplemented with 10% fetal calf serum, 100 IE/ penicillin G, 100 IE /ml streptomycin. Medium and serum were purchased from GibcoBRL (Eggenstein, Germany).

### Production of EAV-specific antisera

Antiserum against EAV was induced in New Zealand white rabbit. The animal was inoculated subcutaneously with 0.5 ml purified EAV. Inoculation was repeated for four times. The experimental protocol is summarized in Table 1. The sensitivity of rabbit antisera was determined by western blot analysis. It was found that the rabbit antiserum raised against EAV is able to recognize viral specific protein at the dilution of about 1:2000 and higher.

### Preparation of viral RNA

Virion RNA and total infected cell RNA was prepared from EAV-infected RK13 cell cultures at 12, 24, 36, and 48 h p.i. using a guanidinium iso-thiocyanate/cesiumchloride procedure based on the method described by Glišin *et al.* (1974). Infected cells or virions from clarified infected cell culture supernatants were dissolved in a 4.0 M guanidinium thiocyanate (GTC) solution. Cellular DNA in the infected cell preparation was sheared by repeatedly passing the solution through a 23-gauge needle. CsCl and sarcosyl (30% aqueous solution) were added to the GTC preparation to final concentrations of 0.15 g/ml and 3.0%, respectively. In volumes of 8 ml the preparation was transferred onto a 3 ml 5.7 M CsCl cushion and centrifuged at 29,000 rpm in a Beckman SW41 rotor for 24 h at 20°C. The supernatant was discarded and the RNA pellet was dissolved in RNase free  $\text{H}_2\text{O}$  to a final concentration of about 10 µg/ml. RNA preparations were stored at -

20°C in 80% ethanol containing 100 mM sodium acetate. As an alternative total RNA of EAV-infected cells was prepared using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the instructions of the manufacturer.

#### First-strand cDNA Synthesis

For each first-strand cDNA synthesis reaction approximately 0.5 µg of purified RNA were pelleted and dissolved in 10 µl RNase free H<sub>2</sub>O containing 20 U RNase inhibitor (Takara Shuzo Co., Ltd., Shiga, Japan). The reaction was prepared in 20 µl volumes using enzymes and reagents from the RNA LA PCR Kit Ver.1.1 (Takara Shuzo Co., Ltd., Shiga, Japan) according to the instructions of the manufacturer. The reaction included 5 mM MgCl<sub>2</sub>, 1 mM of each dNTP, 10 pmol of a specific reverse oligonucleotide primer, and 5 U AMV reverse transcriptase XL. The reaction was incubated in an automated temperature cycling reactor (Genius, Techne, Cambridge, UK) for 2 min at 60°C followed by 15 min at 50°C. Then the temperature was gradually lowered to 42°C at a speed of 1°C/min. As a final step the reaction was incubated for 2 min at 80°C and rapidly cooled to 4°C. RNase free H<sub>2</sub>O was added to the reaction products to obtain a final volume of 100 µl. The first-strand cDNA stocks were stored at -20°C.

#### Oligonucleotides and polymerase chain reaction (PCR)

Specific oligonucleotides were synthesized with an Oligo 1000M DNA Synthesizer (Beckman Instruments GmbH, München, Germany). The properties of the individual oligonucleotides primers are summarized in Table 2. Polymerase chain reaction (PCR) was performed in 100 µl volumes using TaKaRa LA Taq DNA polymerase (supplied with reaction buffer, Takara Shuzo Co., Ltd., Shiga, Japan). Each reaction contained 1.5-2.5 mM MgCl<sub>2</sub>, 12.5 nmol of each dNTP (Boehringer Mannheim Biochemica, Mannheim, Germany), 50 pmol of each oligonucleotide primer, and 1 µl of a first-strand cDNA stock solution (see above). An improved PCR protocol was developed based on a combination of commonly used hot-start and touch-down procedures. Briefly, before adding the dNTP mixture and the DNA polymerase the samples were preheated for 5 min at 94°C and rapidly cooled to 4°C. Then dNTPs and DNA polymerase were added at 4°C and the reaction tubes were directly transferred to a preheated temperature cycling reactor (Genius, Techne, Cambridge, UK) at 94°C. PCR reactions were incubated for 35 cycles under cycling conditions of 94°C for 30 sec, 70-56°C for 1 min (starting at 70°C and decreasing by 0.4°C per cycle), and 72°C for 1-5 min, depending on the size of the expected PCR product. As a final step the reaction mixture was incubated for 7 min at 72°C. Reaction products were analyzed by polyacrylamide slab gel electrophoresis and ethidium bromide staining.

### Molecular cloning of viral cDNA and preparation of plasmid DNA

PCR products representing EAV-specific cDNA sequences were subjected to restriction endonuclease treatment and restriction fragments were purified using preparative low melting point agarose gel electrophoresis. Specific DNA bands were extracted from the gel by a hot phenol procedure followed by gel filtration. Restricted and purified EAV cDNA was inserted into one of the following mammalian expression vectors: pCR3.1, poDNA3.1, pcDNA3.1/His, pDisplay (Invitrogen). Vector plasmids were prepared using restriction endonucleases and purified as described above. In addition, restricted vector DNA was dephosphorylated using calf intestine phosphatase (CIP). Ligation of specific EAV cDNA fragments with expression vector DNA was performed as described previously (Rösen-Wolff *et al.*, 1991). The resulting recombinant plasmid constructs are listed in Table 2. The specificity of the reaction products was confirmed by nucleotide sequence analysis of the insert and flanking vector regions.

### Nucleotide sequence analysis

PCR products were treated with 1 vol phenol:chloroform (5:1) and precipitated with 3 vol 95% ethanol containing 100 mM sodium acetate. The DNA was then washed with 70% ethanol and dissolved in bidistilled water to a final concentration of 20 ng/µl. Plasmid DNA was prepared using the Qiagen tip100 Kit (Qiagen, Hilden, Germany) according to the instructions of the manufacturer. Purified DNA was adjusted in H<sub>2</sub>O to a final concentration of 1 µg/µl. Purified DNA was automatically sequenced with a 373A "Extended" DNA sequencer using the BigDye Terminator-*Taq* cycle sequencing technique (Applied Biosystems GmbH, Weiterstadt, Germany). Each sequencing reaction was performed in a volume of 20 µl containing 100 ng of a PCR product or 0.5 µg of plasmid DNA, 50 pmol of the sequencing primer, and 5 µl of the BigDye Terminator reaction mixture. The cycle sequencing reaction was incubated for 28 cycles in an automated temperature cycling reactor (GeneE, Techne, Cambridge, UK) under cycling conditions of 96°C for 30 sec and 60°C for 4 min per cycle. The samples were prepared for electrophoresis as described by the manufacturer. The electrophoresis of the samples was carried out on a 36-well 48-cm WTR (well to read) polyacrylamide gel. The nucleotide sequences obtained from individual sequencing reactions were assembled using the Sequence Navigator software (version 2.1, Applied Biosystems GmbH, Weiterstadt, Germany). Nucleotide and amino acid sequences were compared to current GenBank, EMBL, and SwissProt database sequence entries using the BLAST service of the National Center for Biotechnology Information (National Library of Medicine, Bethesda, MD, USA). Physico-chemical properties of proteins were determined and conserved sequence motifs were identified with the PHYSCHEM and PROSITE

programs included in the PC/Gene software (release 6.85, A. Bairoch, University of Geneva, Switzerland). The ClustalX program (version 1.64b) (Thompson *et al.*, 1997) was used to generate multiple sequence alignments.

#### Preparation of viral RNA and Northern blot analysis

Total cellular RNA was isolated at different times after infection using the guanidium/cesium chloride method as described previously (Rösen-Wolff *et al.*, 1988, 1989; Rösen-Wolff and Darai, 1991). The northern blot analyses of these RNAs were carried out using formaldehyde agarose gel (1%) electrophoresis as described elsewhere (Rösen-Wolff *et al.*, 1988, 1989; Rösen-Wolff and Darai, 1991).

#### DNA vaccination of animals

The immunogenic potential of the EAV translation units were investigated *in vivo* using BALB/c mice that were administered with DNA of the constructed expression vectors harboring and expressing the cDNA of the individual ORFs of EAV. The BALB/c mice were injected with about 100 µg/DNA diluted in 100 µl PBS. The DNA was injected subcutaneously and into the tibialis cranialis muscle (*Musculus gastrocnemius*) and with a 27 gauge needle. The mice were boosted 3 to 5 times with the same quantities of DNA and under the same conditions at about two week intervals. Control mice received the same amount of parental expression vectors via an identical route and frequency.

#### Neutralization test

Neutralization tests were carried out by diluting EAV-specific mouse or rabbit sera with PBS (1:2 to 1:1024) in a Falcon microtiter plate. Serum dilutions (50 µl) were mixed with 100 TCID<sub>50</sub> of EAV (50 µl). The serum-virus mixture was incubated in a 5% CO<sub>2</sub>-air atmosphere at 37°C for 2 h. Subsequently, 5 x 10<sup>3</sup> RK13 cells in suspension were added to each sample of the serum-virus mixture. After 12 h the infected cultures were overlayed with BME containing 10% FCS and 0.5% carboxymethylcellulose. Then the cultures were incubated for 3-4 days at 37°C in a 5% CO<sub>2</sub>-air atmosphere. Titers of infectious units were determined after staining with 1% crystal violet.

#### Immunoblot analysis

Confluent monolayers of cells were harvested by scraping the cells from the culture well, petri dishes, and/or flasks after being washed three times with PBS (pH 7.2). The final cell pellet

was resuspended in distilled water. Protein concentration was measured under the standard method (Bradford, 1976). Samples were dissolved in an equal volume of lysis buffer (0.01 M Tris HCl, 10% glycerol, 2% SDS, 5%  $\beta$ -mercaptoethanol, 0.1% (w/v) bromophenol blue, pH 8), heated for five minutes at 95°C, and subjected to SDS-PAGE according to the method of Laemmli (1970). Proteins derived from infected and transfectant cells, as well as recombinant N protein were separated by SDS-PAGE and electroblotted onto nitrocellulose filters using semi-dry electroblotting chambers (Renner, Dannstadt, Germany). Transfer efficiency was monitored by Ponceau staining (Sigma, Munich, Germany). Filters were blocked for 1 h and incubated with a 1:1000 and 1:2000 dilution of the rabbit antisera mentioned above. Alkaline phosphatase conjugated antibodies (anti rabbit or mouse Ig-AP, Boehringer Mannheim, Germany) were used to detect interaction of the rabbit or mouse antiserum with EAV protein.

#### Immunofluorescence assay

Indirect Immunofluorescence assay was performed essentially as described earlier (Welzel *et al.*, 1998). Briefly, RK13 cell lines were seeded on tissue chamber slides (Nunc Inc., Naperville, USA). The monolayer cell culture were infected with EAV at the MOI of 2 PFU/cell and 48 h after infection the cells were fixed with acetone-methanol and the slides were stored at -20°C. Fluorescein isothiocyanate (FITC)-conjugated anti-rabbit or mice IgG (F(ab')<sub>2</sub> fragment immunoglobulin (Boehringer, Mannheim, FRG) was used as second antibody. In addition, rhodamine B-isothiocyanate (Merck, Darmstadt, Germany) was used at a final concentration of 10 ng/ $\mu$ l for counterstaining of the cells, together with the second antibody.

#### Enzyme linked immunosorbent assay (ELISA)

Polysorp F8 Microtiter Plates (Nunc, Wiesbaden, Deutschland) were coated with 50  $\mu$ l EAV Protein (EAV+Host (RK13)) at a concentration of 2  $\mu$ g x ml<sup>-1</sup> in PBS (+ 0.05% N<sub>3</sub>Na) over night at room temperature, followed by three cycles of washing with H<sub>2</sub>O and then postcoated with 300  $\mu$ l Blocking Buffer (0.017 M Na<sub>2</sub>B<sub>4</sub>O<sub>7</sub> x 10 H<sub>2</sub>O, 0.12 M Na Cl, 0.05 % Tween 20, 1 mM EDTA, 5 % BSA, 0.05 % NaN<sub>3</sub>) for 3h at 28°C followed by three cycles of washing with H<sub>2</sub>O. For the assay, the following reagents were successively used: rabbit anti EAV serum at a reciprocal dilution up to 16000 or mouse anti EAV serum at a reciprocal dilution up to 800. The dilutions were made in Sample buffer POD (DADE Behring, Marburg, Deutschland). After three cycles of washing with 300  $\mu$ l/well, horse-radish peroxidase labeled rabbit-anti IgG second antibody or the horse-radish peroxidase labeled mouse-anti IgG second antibody (Boehringer,

Mannheim, Deutschland) at a predetermined optimum dilution of 1:3000 each was added. The dilutions were made in Blocking buffer. Incubation steps were done for 1h at 28°C, each followed by three cycles of washing with 300 µl/well Washing Buffer (DADE Behring, Marburg, Deutschland). Color was developed by adding 200 µl/well of freshly prepared Buffer/Substrate TMB and Chromogen TMB (10:1) (DADE Behring, Marburg, Deutschland). The assay was stopped after 30 min by the addition of 50 µl/well Stopping Solution POD (DADE Behring, Marburg, Deutschland) and read according to standard procedures at 450 nm on an automatic ELISA reader (MR5000, DYNATECH, Denkendorf, Deutschland).

#### Lymphocyte activation assay

A Lymphocyte activation assay for the eventual use of the cellular response of the immunized mice was established. BALB/c mouse, female, anti-mouse CD3e (500A2, PharMingen, Cat. No. 01511D), anti-mouse CD69 (H1.2F3, PharMingen, Cat. No. 01505B), Geys buffer (140 mM NH<sub>4</sub>Cl, 2.7 mM KCl, 6.5 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.5 mM KH<sub>2</sub>PO<sub>4</sub>, 0.7 mM CaCl<sub>2</sub>, 0.5 mM MgCl<sub>2</sub>), FACS buffer (PBS, 2% FCS, 0.01% NaN<sub>3</sub>), Polystyrene round bottom tubes (Falcon, Becton Dickinson, Cat. No. 2052), and six well plate (TPP, Cat. No. 9206) were used. The measurement of the samples was performed using FACScan (Becton Dickinson), five measurable parameters: three high performance secondary photomultiplier with bandpass filter: 530 nm for fluorescein isothiocyanate (FITC), 585 nm for phycoerythrin (PE) and 650 nm (red fluorescence), forward and side scatter.

One mouse was killed, splenectomy was performed and spleen was in a sterile tube with 1x BME medium. Spleen was passed through a sterile homogeniser for intact spleen cell separation. The homogeniser was washed with 1x BME medium. The solution was centrifuged for 3 min with 1500 rpm. The pellet was dissolved in 1.5 ml Geys buffer. After that the solution was centrifuged for 3 min with 1500 rpm. The pellet was dissolved in 9 ml BME medium with 10% FCS. One 6 well plate was filled with 1 ml 1x BME medium with 10% FCS, in 3 wells of the plate additional 1 µg/ml anti-mouse CD3e was added. 1.5 ml cell solution was added to each well. The 6 well plate was incubated at 37°C, 5% CO<sub>2</sub> for three days. Solution with activated and not activated cells were each divided in four Polystyrene round bottom tubes. The tubes were filled with FACS buffer and centrifuged for 3 min with 1500 rpm. The supernatant was discarded and the pellet was resuspended in two of four tubes with 100µl anti-mouse CD3e (1:100 diluted in FACS buffer) and incubated for 30 minutes at 4°C. For double fluorescence the cells were centrifuged for 3 min with 1500 rpm, the pellet was resuspended with 100µl anti-mouse CD69

(1:100 diluted in FACS buffer) and incubated for 30 minutes at 4°C again. The cells were centrifuged for 3 min with 1500 rpm, the pellet was resuspended in 2 ml FACS buffer, centrifuged again for 3 min with 1500 rpm, and resuspended in 500 µl FACS buffer each, vortexed and measured in the FACScan.

#### Computer-assisted sequence analysis

Nucleotide sequences were compiled using the ABI sequence navigator software version 1.2. Nucleotide and amino acid sequences were analyzed using the PC/GENE program release 6.85 (Intelligenetics Inc. Mountain View, California, U.S.A.) and OMIGA program release 11.3 (Oxford Molecular Group Ltd., Oxford, UK).

## RESULTS

#### Establishment of a cell culture system for virus propagation, isolation of virus particles, and extraction of viral RNA

The equine arteritis virus (EAV) used in this study was grown and propagated on rabbit kidney cells (RK-13). Virions of EAV were prepared, purified, and the viral RNA was extracted.

#### Generation of specific rabbit antisera against whole virus

In order to specifically detect viral gene products by serological assays a polyclonal hyperimmune rabbit antiserum was raised against complete EAV virion components in New Zealand white rabbits. The protocol of the immunization experiment is given in Table 1. The titer of the antiserum was found to be >1:2000 as determined by Western Blot analysis.

#### Generation of rabbit antisera against synthetic polypeptides

The biophysical analysis of the nucleocapsid protein (ORF 7) of EAV indicated that a strong antigenic domain was located within the first 38 amino acids of the ORF 7 gene product. In order to investigate the immunogenic potential of the N-terminus of the viral nucleocapsid protein a synthetic polypeptide corresponding to amino acid residues 1-38 was synthesized and linked to keyhole limpet hemocyanine (KLH) and used to raise polyclonal monospecific antiserum in rabbits. The antiserum was found to detect specific EAV gene product in Western Blot assay at a dilution of 1:200.



### Amplification of viral genes by RT-PCR and molecular cloning of viral genes in expression vectors

The EAV genome consists of about 12.287 nucleotides with a short 3'poly- (A) tail. For molecular cloning of the viral genome specific oligonucleotides were synthesized (Table 2) and purified viral RNA was used in 3'-RACE experiments to generate a cDNA bank from the genomic RNA including cDNA of viral mRNA transcripts. The amplified cDNA fragments include the viral open reading frames (ORFs) 2 to 7, which encode the small glycoprotein, nonstructural proteins, large glycoprotein, membrane protein, and nucleocapsid protein, respectively (Table 3). The viral genes were molecularly cloned into a suitable expression vector (*pCR3.1*, *pDisplay*, and *pcDNA3.1/His A, B, C; Invitrogen*). The identity of the cDNA of the individual viral ORFs was confirmed by nucleotide sequence analysis. The properties of the constructed mammalian expression vectors harboring and expressing EAV-specific cDNA of the individual viral translation units are summarized in Table 3.

As an additional goal for the development of efficient EAV DNA vaccines in future it is envisaged to construct expression vectors harboring and expressing overlapping or full-length viral cDNA. An expression library of cloned high molecular weight viral cDNA was constructed spanning the entire viral genome. Using optimized long-range RT-PCR protocols a number of overlapping viral cDNA fragments ranging from 2,000 to over 8,500 bp were generated. The identities of the RT-PCR products were confirmed by nucleotide sequence analysis. This library is essential to establish full-length cDNA clones expressing the entity of viral antigens simultaneously in *in vivo* and *in vitro* when necessary. The properties of the individual RT-PCR products are summarized in Table 4.

### DNA vaccination of mice with different vector constructs and evaluation of the corresponding immune response

#### DNA vaccination of mice with vector construct expressing viral ORFs 5 and 7

Balb/c mice were used as a model system for the evaluation of the immune responses against individual gene products of EAV raised by administration of recombinant plasmid DNA. The capability of the expression vectors harboring and expressing EAV ORF5 and ORF7 that encode the viral nucleocapsid protein and the viral major glycoprotein to induce immune response in the mouse system was investigated. The animals were inoculated subcutaneously and intramuscularly for five times with 100 µg of the particular DNA in 14-day intervals. Analysis of the individual mouse sera revealed that the administered DNAs of the vectors expressing both

viral gene products are able to induce significant immune response in mice as tested by neutralization test (NT). The results of these studies are summarized in Table 5 and are shown in Fig. 3 for ORF 5. A significant immune response was detected when the animals were immunized with the recombinant plasmids pCR3.1-EAV-O7-BX-C3 and pCR3.1-EAV-O5-C14 harboring ORFs 7 and 5 coding for viral nucleocapsid protein and large envelope glycoprotein ( $G_L$ ), respectively. As shown in Table 5 it was found that the recombinant plasmids pCR3.1-EAV-O7-BX-C3 are able to raise antibodies against EAV in mice (80 % immune response) as detected by Neutralization Test. Similar results (50-70 % immune response) were obtained in two independent experiments when the animals were inoculated with the recombinant plasmids pCR3.1-EAV-O5-BX-C14 (Table 5 and Fig. 3A and B).

In the next step of this investigation the animals were administered with the DNAs of the recombinant plasmids pCR3.1-EAV-O7-BX-C3 and pCR3.1-EAV-O5-C14 simultaneously under the same conditions described above. The results of this study that is in agreement with the corresponding results obtained from the analysis of the gene products of the EAV ORFs 7 and 5 (Table 5 and Fig. 3) are summarized in Table 5 and shown in Fig. 4. These data indicate that the native DNA of recombinant plasmids pCR3.1-EAV-O7-BX-C3 (harboring ORF 7) and pCR3.1-EAV-O5-C14 (harboring ORF 5) is able to express the corresponding gene products (viral nucleocapsid protein and large envelope glycoprotein ( $G_L$ )) *in vivo*.

The results of these studies unambiguously underline that the gene products of the EAV ORFs 7 and 5 are suitable candidates for development of a DNA vaccine protecting an EAV infection.

#### DNA vaccination of mice with vector construct expressing viral ORFs 5, 7, and IL-2

In order to determine whether or not the IL-2 is able to enhance the immunogenic potential of the gene product of the EAV ORFs 5 and 7 by DNA vaccination the following experiments were performed. An expression vector (pWS2ms) containing the murine IL-2 expression cassette (kindly provided by Dr. W. Schmidt, Intercell, Vienna) was co-inoculated with two novel constructed recombinant plasmids pDP-EAV-O7-BgS-C2 and pDP-EAV-O5-C1 harboring and expressing the EAV ORFs 7 and 5 that encode the viral nucleocapsid protein and large envelope glycoprotein ( $G_L$ ), respectively. The construction of these expression vectors based on pDisplay system that is similar to pCR3.1. This system additionally allows the cell surface expression of inserted epitopes by fusion to an N-terminal signal peptide and a C-terminal transmembrane domain. Balb/c mice were inoculated subcutaneously and intramuscularly for four times with 100  $\mu$ g of each of the vector constructs in 14-day intervals. Under the conditions used an enhanced EAV-specific immune response in mice was detectable as shown in Table 5.

DNA vaccination of mice with vector construct expressing viral ORFs 5 and 6

The EAV consists of an unglycosylated membrane protein (M, 17 kDa, gene product of ORF 6). It was of particular interest to proof whether or not this viral gene product can eventually influence and/or enhance the immunogenic potential of the gene products of EAV ORF 5 *in vivo*. A recombinant plasmid pCR3.1-EAV-O6-BE-C3 was constructed in which the translation unit of the EAV ORF 6 was inserted into the corresponding site of the expression vectors pCR3.1. Balb/c mice were inoculated with recombinant plasmids pCR3.1-EAV-O6-BE-C3 and pCR3.1-EAV-O5-C14 under the conditions described above. The sera obtained from the Balb/c mice (pre and post DNA vaccination) were analyzed using neutralization test and the results are given in Table 5 and shown in Fig. 6. A significant enhanced potential of the gene product of the EAV ORF 6 on the functional activities of the viral large envelope glycoprotein (gene product of EAV ORF 5) *in vivo* was not observed.

DNA vaccination of mice with vector construct expressing viral ORFs 3 and 4

Although the functional activity of the gene products of two EAV ORFs 3 and 4 is not known, the eventual activity of these viral proteins *in vivo* was investigated. Two recombinant plasmids pCR3.1-EAV-O3-BX-C1 and pCR3.1-EAV-O4-BX-C3 was constructed (Table 3) in which the cDNA of the EAV ORFs 3 and 4 were inserted into the corresponding site of the expression vector pCR3.1. The sera obtained from the Balb/c mice (pre- and post DNA-vaccination) that were inoculated with these recombinant plasmids were analyzed using neutralization test. The results of these studies are summarized in Table 5 and shown in Fig. 7 and 8 for the gene products of EAV ORFs 3 and 4, respectively. No neutralizing antibodies were detected in the immunized Balb/c mice under the conditions used.

DNA vaccination of mice with vector construct expressing EAV N-terminal hydrophilic ectodomain of large envelope glycoprotein (G<sub>L</sub>)

Balasuriya and coworkers (1995) found that the neutralization determinants of EAV are located on the gene product of ORF 5 (large envelope glycoprotein) within the N-terminal ectodomain (amino acid positions 1-121; Balasuriya *et al.*, 1997). Accordingly, a novel expression vector based on pcDNA3.1/His system was constructed. This vector is similar to pCR3.1, but allows the specific detection and purification of the expressed fusion proteins using N-terminal amino acid sequence tags. The strategy of this experiment is shown in Fig. 2. A recombinant plasmid was constructed and termed pC31-HIS-EAV-O5-del-121. The insert of this expression vector possesses the coding region of EAV ORF 5 corresponding to amino acids 1-121 of the viral large

envelope glycoprotein which was inserted into the corresponding sites of the parental vector pcDNA3.1-HIS-C (Table 5 and Fig. 2). Balb/c mice were inoculated with recombinant plasmid pC31-HIS-EAV-O5-del-121 under the conditions described above. The sera obtained from the Balb/c mice (pre and post DNA vaccination) were analyzed using neutralization test. As shown in Table 5 and Fig. 9 it was found that the majority of the DNA vaccinated animals (90 %) developed a significant neutralizing antibody against EAV.

DNA vaccination of mice with vector constructs expressing viral membrane and glycoproteins in combination with IL-2 gene expression

As described above, it had been shown that the viral N-glycosylated major membrane protein (G<sub>L</sub>), the N-glycosylated minor membrane protein (G<sub>S</sub>, 25 kDa), and unglycosylated membrane protein (M, 17 kDa) are able to develop significant immune response *in vivo* as detected by DNA vaccination of Balb/c mice. Furthermore, the enhancer function of IL-2 gene expression during DNA vaccination is known. However, an intermolecular interaction of these proteins during gene expression *in vivo* e.g. by DNA vaccination is not known, so far. Therefore it was of particular importance to proof whether or not these proteins are capable to induce an adequate immune response by simultaneous expression *in vivo*.

Four recombinant plasmids pCR3.1-EAV-O6-BE-C4, pCR3.1-EAV-O2-BX-C5, pC3.1-EAV-O5-BX-C14, and pWS2ms were used. These vector constructs are able to express viral membrane protein, glycoproteins G<sub>S</sub>, G<sub>L</sub> and mice interleukin 2, respectively. Balb/c mice were inoculated with a DNA mixture containing the four recombinant plasmids under the standard conditions described above. The sera obtained from the Balb/c mice (pre- and post DNA vaccination) were analyzed using neutralization test and the corresponding data are summarized in Table 5 and shown in Fig. 10. Under the conditions used in this experiment 90% of the DNA vaccinated mice developed significant immune response against EAV.

DNA vaccination of mice with vector constructs expressing viral small glycoprotein and the gene product of the ORF 4

The capability of the EAV small glycoprotein (Gene products of ORF 2) and the gene product of ORF 4 to develop immune response *in vivo* was investigated by DNA vaccination of Balb/c mice. Two recombinant plasmids pCR3.1-EAV-O2-BX-C5 and pCR3.1-EAV-O4-BX-C3 were used. These vector constructs are able to express viral small glycoproteins G<sub>S</sub> and the gene product of EAV ORF 4, respectively. Balb/c mice were inoculated with a DNA mixture containing the two recombinant plasmids under the standard conditions described above. The

sera obtained from the Balb/c mice (pre and post DNA vaccination) were analyzed using neutralization test. As shown in Fig. 11 and Table 5, it was found that about 50% of the DNA vaccinated animals developed NT-titer against EAV.

#### Logistics for DNA vaccination of horses

In addition to the objective of our studies, it was necessary to develop and establish a novel screening system for detection of humoral and cellular immune response in vaccinated horses. An enzyme linked immunosorbent assay (ELISA) and a lymphocyte activation assay were established as described in the session of material and methods. Furthermore, it was necessary to prepare a convenient kit for vaccination of horses with the constructed expression vectors harboring and expressing the viral gene products of ORFs 2 to 7.

#### Development of a novel Enzyme linked immunosorbent assay (ELISA)

Polysorp F8 Microtiter Plates were coated with Protein of EAV infected RK-13 cells at a concentration of  $2 \mu\text{g} \times \text{ml}^{-1}$ . In this system antibodies against EAV (rabbit, mouse, and horse serum, etc.) can be detected. Horse-radish peroxidase labeled rabbit-anti IgG or horse-radish peroxidase labeled mouse-anti IgG served as second antibody. The color was developed by adding Buffer/Substrate TMB and Chromogen TMB and the adsorbance was determined according to standard procedures at 450 nm on an automatic ELISA reader (for detail see session material and methods). An example of the results of this analysis is shown in Fig. 12. The viral antigens prepared for ELISA can be used for performance of about 20,000 ELISA assays.

#### Development of a Lymphocyte activation assay

A Lymphocyte activation assay for the eventual use of the cellular response of the immunized mice was established. BALB/c mouse, female, anti-mouse CD3 $\epsilon$  (500A2, PharMingen, Cat. No. 01511D), anti-mouse CD69 (H1.2F3, PharMingen, Cat. No. 01505B), Geys buffer (140 mM NH<sub>4</sub>Cl, 2.7 mM KCl, 6.5 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.5 mM KH<sub>2</sub>PO<sub>4</sub>, 0.7 mM CaCl<sub>2</sub>, 0.5 mM MgCl<sub>2</sub>), FACS buffer (PBS, 2% FCS, 0.01% NaN<sub>3</sub>), Polystyrene round bottom tubes (Falcon, Becton Dickinson, Cat. No. 2052), and six well plate (TPP, Cat. No. 9206) were used. The measurement of the samples was performed using FACScan (Becton Dickinson), five measurable parameters: three high performance secondary photo multiplier with band pass filter: 530 nm for fluorescein isothiocyanate (FITC), 585 nm for phycoerythrin (PE) and 650 nm (red fluorescence), forward and side scatter.

One mouse was killed, splenectomy was performed and spleen was transferred to a sterile tube with 1x BME medium. Intact spleen cells were separated and were washed with 1x BME medium. The solution was centrifuged for 3 min with 1500 rpm. The pellet was dissolved in 1.5 ml Geys buffer. After that the solution was centrifuged for 3 min with 1500 rpm. The pellet was dissolved in 9 ml BME medium with 10% FCS. One 6 well plate was filled with 1 ml 1x BME medium with 10% FCS, in 3 wells of the plate additional 1 µg/ml anti-mouse CD3ε was added. 1.5 ml cell solution was added to each well. The 6 well plate was incubated at 37°C, 5% CO<sub>2</sub> for three days. Solutions with activated and not activated cell were each divided in four Polystyrene round bottom tubes. The tubes were filled with FACS buffer and centrifuged for 3 min with 1500 rpm. The supernatant was discarded and the pellet was resuspended in two of four tubes with 100µl anti-mouse CD3ε (1:100 diluted in FACS buffer) and incubated for 30 minutes at 4°C. For double fluorescence the cells were centrifuged for 3 min with 1500 rpm, the pellet was resuspended with 100µl anti-mouse CD69 (1:100 diluted in FACS buffer) and incubated for 30 minutes at 4°C again. The cells were centrifuged for 3 min with 1500 rpm, the pellet was resuspended in 2 ml FACS buffer, centrifuged again for 3 min with 1500 rpm, and resuspended in 500 µl FACS buffer each, vortexed and measured in the FACScan.

**Table 1:** An example of experimental protocol used for production of polyclonal antibodies against equine arteritis virus in New Zealand white rabbit

<b>Date</b>	<b>Treatment</b>
<b>09.06.1998</b>	Preimmun-serum 10 x 620 µl
<b>10.06.1998</b>	1 <sup>st</sup> inoculation* 0.5 ml/s.c.
<b>24.06.1998</b>	2 <sup>nd</sup> inoculation* 0.5 ml/s.c.
<b>08.07.1998</b>	3 <sup>rd</sup> inoculation* 0.5 ml/s.c.
<b>22.07.1998</b>	4 <sup>th</sup> inoculation* 0.5 ml/s.c. <b>First bleeding (10 ml)</b> <b>Titer by immunoblot analysis = 1:200</b>
<b>30.07.1998</b>	5 <sup>th</sup> inoculation* 0.5 ml/s.c.
<b>11.08.1998</b>	<b>Final bleeding (30 x 1.5 ml)</b> <b>Titer by immunoblot analysis = &gt;1:2000</b>

**Virus:** Equine arteritis virus: Virion in 0.5 ml PBS, purified by sucrose gradient centrifugation

**Animal:** New Zealand white rabbit (ca 2 kg/female)

Table 2: List of oligonucleotide primers that were used for the amplification and molecular cloning of equine arteritis virus cDNA. Artificial sequences containing restriction endonuclease recognition sites (underlined) are shown in boldface letters.

Primer Name	Nucleotide Sequence
P-XbaI-EAV-O5-R1	5'-GGGTCTAGAGTCACCACAAAATGAATCTATGGC-3'
P-BamHI-EAV-O5-F1	5'-GGGGGATCCTGGTACGTTGGGCTCAACGATG-3'
P-XbaI-EAV-O7-R1	5'-GGGTCTAGACCACACAGGAGAATATCCACGTC-3'
P-BamHI-EAV-O7-F1	5'-GGGGGATCCCGCAGTTGGTAACAAGCTTGTCG-3'
P-XbaI-EAV-O5-R1	5'-GGGTCTAGAGTCACCACAAAATGAATCTATGGC-3'
P-BamHI-EAV-O5-F1	5'-GGGGGATCCTGGTACGTTGGGCTCAACGATG-3'
P-BamHI-EAV-O2-F1	5'-GGGGGATCCATTCTGTGATTGATGCAGCGC-3'
P-XbaI-EAV-O2-R1	5'-GGGTCTAGAGGCATATTCATAACCCGTGTGCACTAC-3'
P-BamHI-EAV-O3-F1	5'-GGGGGATCCTTTGACCGGACCGGCCACATGGGTC-3'
P-XbaI-EAV-O3-R1	5'-GGGTCTAGAGTAAGTAAAATTACGAGCCTCTGCAGC-3'
P-BamHI-EAV-O4-F1	5'-GGGGGATCCCCCTTTGTAGATGAAGATCTACGGC-3'
P-XbaI-EAV-O4-R1	5'-GGGTCTAGAGCAATACAATCATAGATAACATCGTTGAGCCC-3'
P-BamHI-EAV-O6-F1	5'-GGGGGATCCCAGCTGAGGTATGGGAGCCATAG-3'
P-EcoRI-EAV-O6-R1	5'-GGGGAATTCATGCGCAGTAGGTCATTGTAGC-3'
P-BamHI-EAV-O5-F1n	5'-GGGGGGATCCTGGTACGTTGGGCTCAACGATG-3'
P-XbaI-EAV-O5-R1n	5'-GGGGGTCTAGAGTCACCACAAAATGAATCTATGGC-3'
P-BamHI-EAV-O7-F1n	5'-GGGGGGATCCCGCAGTTGGTAACAAGCTTGTCG-3'
P-XbaI-EAV-O7-R1n	5'-GGGGGTCTAGACCACACAGGAGAATATCCACGTC-3'
P-SalI-EAV-O5-R1	5'-GGGGTCGACGTCACCACAAAATGAATCTATGGC-3'
P-EcoRI-EAV-O7-R1	5'-GGGGAATTCACACAGGAGAATATCCACGTC-3'
P-EAV-O5-R1	5'-CAACTATGCCGAATTCACGGCC-3'
P-EAV-O5-F1	5'-GGCCGTGAATTCGGCATAAGTTG-3'
P-BamHI-EAV-O2-F1n	5'-GGGGGGATCCATTCTGTGATTGATGCAGCGC-3'
P-XbaI-EAV-O2-R1n	5'-GGGGGTCTAGAGGCATATTCATAACCCGTGTGCACTAC-3'
P-BamHI-EAV-O3-F1n	5'-GGGGGGATCCTTTGACCGGACCGGCCACATGGGTC-3'
P-XbaI-EAV-O3-R1n	5'-GGGGGTCTAGAGTAAGTAAAATTACGAGCCTCTGCAGC-3'
P-BamHI-EAV-O4-F1n	5'-GGGGGGATCCCCCTTTGTAGATGAAGATCTACGGC-3'
P-XbaI-EAV-O4-R1n	5'-GGGGGTCTAGAGCAATACAATCATAGATAACATCGTTGAGCCC-3'



Table 2 continued:

List of oligonucleotide primers that were used for the amplification and molecular cloning of equine arteritis virus cDNA. Artificial sequences containing restriction endonuclease recognition sites (underlined) are shown in boldface letters.

Primer Name	Nucleotide Sequence
P-BamHI-EAV-O6-F1n	5'-GGGGGGGATCCAGCTGAGGTATGGGAGCCATAG-3'
P-EcoRI-EAV-O6-R1n	5'-GGGGGGAATTCATGCGCAGTAGGTCATTGTAGC-3'
P-SalI-EAV-O5-R1n	5'-GGGGGGGTCTGACTGGCTCCCATACCTCAGCTGC-3'
P-BglII-EAV-O7-F1n	5'-GGGGGGGAGATCTATGGCGTCAAGACGATCACGTCCG-3'
P-SalI-EAV-O7-R1n	5'-GGGGGGGTCTGACCGGCCCTGCTGGAGGCGCAAC-3'
P-BglII-EAV-O2-F1n	5'-GGGGGGGAGATCTATGCAGCGCTTTTCTTTCTCATGC-3'
P-SalI-EAV-O2-R1n	5'-GGGGGGGTCTGACCAAAATCTTGCGCCGCGGCAAAG-3'
P-BglII-EAV-O6-F1n	5'-GGGGGGGAGATCTATGGGAGCCATAGATTTCATTTTGTGG-3'
P-SalI-EAV-O6-R1n	5'-GGGGGGGTCTGACTTGTAGCTTGTAGGCTGTGCGCCG-3'
P-EcoRI-EAV-O1-F1n	5'-GGGGGGAATTCATGGCAACCTTCTCCGCTACTGG-3'
P-XbaI-EAV-O1-R1n	5'-GGGGGGTCTAGATCACACGGGCCCAATGACTGAACC-3'
P-AflII-TOEAV-F1	5'-CCCCTTAAGTGCCATATACGGCTCACCACCATATACAC-3'
P-MroI-TOEAV-R1	5'-CCCTTCCGGAGGTTCTTGGGTGGCTAATAACTACTTC-3'
PR-BAM-5102	5'-GGGGGGGATCCAGTACTTTGGC-3'
PF-BAM-5097	5'-GGGGGGGATCCAGCGACACCCGAGGCACG-3'
PR-SPH-8570	5'-GGGGGGGCATGCTGTAGGTGGGCCATGGATCAAGTCC-3'
PF-SPH-8565	5'-GGGGGGGCATGCCACCTGGGCCAAGAAATTGACC-3'
PR-EAV-6959-6946	5'-GCTGGCCGCGTAATGCTGGTTGGG-3'
PF-EAV-5948-5973	5'-CCGTGCTGGATGTGAGGCCGTTACCG-3'
P-EAV-GL-ECTO-F	5'-GGGGGGGATCCATGTTATCTATGATTGTATTGCTATTTC-3'
P-EAV-GL-ECTO-R	5'-GGGGGGTCTAGACAACACAACACTATGCCGAATTCAC-3'
P-pcDNA3.1/HisC-F	5'-ATGGTATGGCTAGCATGACTGGT-3'
P-pcDNA3.1/HisC-R	5'-CAAACAACAGATGGCTGGCAACT-3'

Table 3: Properties of the constructed mammalian expression vectors harboring EAV-specific cDNA of the individual translations units.

Construct Name	EAV-Specific Insert (Nucleotide Position)	Expression Vector (Restriction Sites)	Oligonucleotide Primers
pCR3.1-EAV-O2-BX	ORF 2: small glycoprotein (9807-10490)	pCR3.1 ( <i>Bam</i> HI / <i>Xba</i> I) G/GATCC / T/CTAGA	P-BamHI-EAV-02-F1n P-XbaI-EAV-02-R1n
pCR3.1-EAV-O3-BX	ORF 3 (10289-10780)	pCR3.1 ( <i>Bam</i> HI / <i>Xba</i> I) G/GATCC / T/CTAGA	P-BamHI-EAV-03-F1n P-XbaI-EAV-03-R1n
pCR3.1-EAV-O4-BX	ORF 4 (10683-11141)	pCR3.1 ( <i>Bam</i> HI / <i>Xba</i> I) G/GATCC / T/CTAGA	P-BamHI-EAV-04-F1n P-XbaI-EAV-04-R1n
pCR3.1-EAV-O5-BX	ORF 5: large glycoprotein (11129-11896)	pCR3.1 ( <i>Bam</i> HI / <i>Xba</i> I) G/GATCC / T/CTAGA	P-BamHI-EAV-05-F1n P-XbaI-EAV-05-R1n
pDP-EAV-O5-BgS	ORF 5: large glycoprotein (11129-11896)	PDisplay ( <i>Bgl</i> II / <i>Sal</i> I) A/GATCT / G/TCGAC	P-BglII-EAV-05-F1n P-SalI-EAV-05-R1n
pCR3.1-EAV-O6-BE	ORF 6: membrane protein (11884-12372)	pCR3.1 ( <i>Bam</i> HI / <i>Eco</i> RI) G/GATCC / G/AATTC	P-BamHI-EAV-06-F1n P-EcoRI-EAV-06-R1n
pCR3.1-EAV-O7-BX	ORF 7: nucleocapsid protein (12296-12628)	pCR3.1 ( <i>Bam</i> HI / <i>Xba</i> I) G/GATCC / T/CTAGA	P-BamHI-EAV-07-F1n P-XbaI-EAV-07-R1n
pDP-EAV-O7-BgS	ORF 7: nucleocapsid protein (12296-12628)	PDisplay ( <i>Bgl</i> II / <i>Sal</i> I) A/GATCT / G/TCGAC	P-BglII-EAV-07-F1n P-SalI-EAV-07-R1n
pCR3.1-HIS-EAV-O5-del-1-121-BX	partial ORF 5: C- terminal ectodomain of large glycoprotein (11129-11492)	peDNA3.1/HIS C ( <i>Bam</i> HI / <i>Xba</i> I) G/GATCC / T/CTAGA	P-EAV-GL-ECTO-F P-EAV-GL-ECTO-R

Table 4: Properties of the equine arteritis virus specific cDNA obtained by long range RT-PCR

PCR product (viral ORFs)	Nucleotide Position	Size of Viral cDNA Fragment bp	Site (s) of Restriction Enzyme (s)	Oligonucleotide Primers
PCR-EAV-1-5102 (truncated ORF 1 a*)	1-5102	5102	<i>AflII/BamHI</i> C/TTAAG / G/GATCC	P-AflII-TOEAV-F1 & PR-BAM-5102
PCR-EAV-5097-8570 (Part of ORF1 a & b*)	5097-8570	3473	<i>BamHI/SphI</i> G/GATCC / GCATG/C	PF-BAM-5097 & PR-SPH-8570
PCR-EAV-8565-12687 (Part of ORF1 b & 2-7**)	8565-12687	4122	<i>SphI/MroI</i> GCATG/C / T/CCGGA	PF-SPH-8565 & P-Mro I-TOEAV-R1
PCR-EAV-1-8570 (ORF 1 a & truncated 1b*)	1-8570	8570	<i>AflII/SphI</i> C/TTAAG / GCATG/C	P-AflII-TOEAV-F1 & PR-SPH-8570
PCR-EAV-5097-12687 (Part of ORF1a* -7**)	5097-12687	7590	<i>BamHI/MroI</i> G/GATCC / T/CCGGA	PF-BAM-5097 & P-Mro I-TOEAV-R1
PCR-EAV-1-6959 (ORF1 a & part of 1b*)	1-6959	6959	<i>AflII</i> C/TTAAG	P-AflII-TOEAV-F1 & PR-EAV-6959-6946
PCR-EAV-5948-12687 (Part of ORF1b* -7**)	5948-12687	6739	<i>MroI</i> T/CCGGA	PF-EAV-5948-5973 & P-Mro I-TOEAV-R1

\* ORF1: ORF 1a: 208-5391, ORF 1b: 5388-9734 nucleotides

\*\* ORF2-7: 9807-12628 nucleotides

Table: 5: Summary of the results obtained by DNA immunization of mice using different gene products of equine arteritis virus

Experiment (No. of animals)	Expression Vector (ORF/Gene product)	Average of NT-AB <sup>a</sup> titer minimum/ maximum		% of Immun- response <sup>b</sup>
		PIS	PVS	
<u>MV-00-01(10)</u> 07/27/1998 to 10/19/1998	pCR3.1	<1:10	<1:10	0
<u>MV-07-01(10)</u> 07/27/1998 to 10/19/1998	pCR3.1-EAV-07-BX-C3 (ORF 7/nucleocapsid protein =NP)	<1:10/1:10	1:20/1:640	80
<u>MV-05-01(10)</u> 08/29/1998 to 11/11/1998	pCR3.1-EAV-05-BX-C14 (ORF 5/large envelope glycoprotein = G <sub>L</sub> )	<1:10	1:10/1:160	70
<u>MV-05-02(10)</u> 12/07/1998 to 03/20/1999	pCR3.1-EAV-05-BX-C14 (ORF 5/ G <sub>L</sub> )	<1:10	1:10/1:160	50
<u>MV-57-01(10)</u> 09/11/1998 to 11/23/1999	pCR3.1-EAV-05-BX-C14 (ORF 7/NP) pCR3.1-EAV-07-BX-C3 (ORF 5/G <sub>L</sub> )	<1:10	1:10/1:80	30/70 <sup>c</sup>
<u>MV-57IL2-1(10)</u> 10/26/1998 to 01/25/1999	pDP-EAV-05-BgS-C1 (ORF 7/NP) pDP-EAV-07-BgS-C2 (ORF 5/G <sub>L</sub> ) pWS2ms (IL2)	<1:10/1:10	1:10/1:80	40/80 <sup>c</sup>
<u>MV-56-01(10)</u> 12/07/1998 to 02/08/1999	pCR3.1-EAV-05-BX-C14 (ORF 7/NP) pCR3.1-EAV-06-BE-C4 (ORF 6/membrane protein)	<1:10	1:10/1:80	20/70 <sup>c</sup>
<u>MV-03-01(10)</u> 12/21/1998 to 03/22/1999	pCR3.1-EAV-03-BX-C1 (ORF 3/?)	<1:10	<1:10/1:10	0
<u>MV-04-01(10)</u> 12/22/1998 to 03/22/1999	pCR3.1-EAV-04-BX-C3 (ORF4/?)	<1:10	<1:10/1:20	0
<u>MV-05d-01(10)</u> 12/29/1998 to 03/22/1999	pC3.1-HIS-EAV-05-del-121 (amino terminus of large envelope glycoprotein; the first 121 amino acids)	<1:10	1:20/1:160	90/100 <sup>c</sup>

<u>MV-</u> <u>256IL-2-1</u> <u>(10)</u> <u>02/12/1999</u> <u>to</u> <u>04/07/1999</u>	<p>PCR3.1-EAV-O2-BX-C5 (ORF 2/small glycoprotein)</p> <p>PCR3.1-EAV-O5-BX-C14 (ORF 5/G<sub>L</sub>)</p> <p>PCR3.1-EAV-O6-BE-C4 (ORF 6/membrane protein)</p> <p>pWS2ms (IL2 gene)</p>	<1:10/1:10	1:10/1:80	70/90 <sup>c</sup>
<u>MV-</u> <u>99/5(10)</u> <u>05/14/1999</u> <u>to</u> <u>07/09/1999</u>	<p>PCR3.1-EAV-O2-BX-C5 (ORF 2/small glycoprotein)</p> <p>PCR3.1-EAV-O4-BX-C3 (ORF4/?)</p>	<1:10/1:10	<1:10/1:80	40/50 <sup>c</sup>

<sup>a</sup> Neutralizing antibodies<sup>b</sup> Neutralizing titer 1:20 excluded<sup>c</sup> Neutralizing titer 1:20 included

PIS: Preimmune serum; PVS: Post vaccination serum

<sup>?</sup> Protein of unknown function

## **Example 2 DNA vaccination of horses**

### **Preparation of a DNA vaccine kit for application in horses**

A convenient kit for vaccination of horses harboring the individual constructed recombinant plasmid (10 mg DNA) expressing the viral gene products of ORFs 2 to 7 was prepared. The properties of the DNA vaccination kit are summarized in Table 6.

In order to proof the ability of the expression of EAV cDNAs of ORFs 2 and 5 to 7 in the autologous animal system horse it was necessary to screen the horse sera prior to immunization trial. The selection of the suitable animals was based on the results obtained from the analysis of the horse sera. These studies allowed detecting the existence of a previous naturally occurred EAV infection in horses. Five sera were obtained from Prof. Dr. H. Müller (Institut für Virologie, Veterinärmedizinische Fakultät, Universität Leipzig). The horse sera were analyzed by neutralization assay and ELISA as described above. The results of these experiments for sera obtained from Leipzig are summarized in table 7. In contrast, it was found that three horses from the animal farms of Leipzig did not developed specific antibodies against an EAV infection. Consequently, it seem to be rational that the animals from the later farm can be considered for evaluation of EAV DNA vaccine in its natural host.

### **Preparation of autologous skin fibroblasts**

Two skin biopsies were performed from each of the five horses involved in the study (Daggy, Frieda, Friedrich, Jessy, Nelke). Skin samples were cut into (5-8) slices and attached to cell culture flasks. After a 30 min incubation at 37°C and 5% CO<sub>2</sub>, skin samples were cultured in Dulbecco's modified eagles medium (DMEM) containing 10% fetal calf serum (FCS), antibiotics and antimycotics. Fibroblasts growing out of the skin samples were passaged twice and extended to 2x10<sup>7</sup> cells. From each horse cells were frozen in liquid nitrogen (N<sub>2</sub>) in aliquots containing at least 2x10<sup>6</sup> cells.

Several aliquots were thawed and cultured in DMEM containing 10% FCS and antibiotics to verify the competence of these cells for further growth. Cells were passaged more than 20 times. For transfection experiments, however, cell of passage 15 and more proved to be not suitable.

### Purification of plasmid DNA

Plasmids were obtained from Boehringer Ingelheim (named pCR3.1-BAV-02-BX-C3), pCR3.1-BAV-05-BX-C14, and pCR3.1-EAV-07-BX-C3). After selection of ampicillin resistant e.coli K12 colonies, bacterial cultures containing the different plasmids were grown to large scale. From each plasmid 500µl DNA at a concentration of 1.5µg/µl were isolated and stored at -20°C for the transfection experiments.

### Transfection experiments

Transfection experiments were initially performed in 12 and 24 well plates. Titration experiments in the 24 well plate format using cell numbers between  $1.5 \times 10^4$  and  $3 \times 10^5$  per well revealed that  $1.25 \times 10^5$  cells seeded in each well were almost confluent (85%) within 24 hours.

#### a) Lipofection as transfection reagents

Titration experiments performed in a 24 well plate format using 5-20µl/well Lipofectin showed that amounts >15µl were toxic to the cells. Titration experiments using different amounts (5-80µg/well) of DNA with 12.5 µl Lipofectin revealed that concentrations of 80µg DNA showed the highest transfection efficiencies. However, the efficiency in these transfection experiments did not exceed 10%.

#### b) LipofectAMINE as transfection reagent

In order to increase the transfection efficiency, transfection experiments were performed using additional transfection reagents (LipofectAMINE, LipofectAMINE *plus*, DMRIE). Compared to the results of the transfection experiments using Lipofectin, DMRIE did not result in higher number of transfected cells. LipofectAMINE showed to be more toxic to the cells, but the transfection efficiencies were higher. The recommendation of the supplier to use in addition to LipofectAMINE the '*plus*' reagent did not increase the transfection rate. Therefore, LipofectAMINE seems to be the reagent of choice to transfect the cultured primary horse skin fibroblasts.

In the initial experiments using 7.5µl LipofectAMINE and 5µg of DNA, about 15% of the target cells were transfected. Currently transfection experiments are being performed to further increase the transfection efficiency. So far, the transfection efficiency is about 20%.

### Isolation of peripheral blood lymphocytes

Three weeks and one day before the initial immunisation, 50ml of heparinised blood was collected from each horse by jugular venipuncture. The blood was centrifuged at 400 x g for 5 min and the plasma was removed. Blood cells (10ml) were resuspended with phosphate buffered saline (PBS) to a volume of 25ml, layered on a Ficoll Hypaque gradient (15ml) and centrifuged at 400 x g for 30min. The PBMC were collected from the interface, washed twice in PBS, counted and frozen in aliquots in N<sub>2</sub> in 10% DMSO and 90% FCS.

To test the viability of the PBMC, cells were thawed, cultured in Isocove's modified Eagles medium (IMEM) containing 10% FCS and antibiotics. PBMC were stimulated for two days with 2.5µg/ml pokeweed mitogen and cultured thereafter in the presence of 200U of human recombinant IL-2. PBMC showed a good proliferation, were grown to 2x10<sup>6</sup> cells/ml and were frozen again as aliquots in N<sub>2</sub>.

### Detection of EAV-specific antibodies

Serum was collected 4 months, three weeks and one day before the initial immunisation. Serum of the first timepoint was investigated at the Institute of Medical Virology (Prof. Darai) in Heidelberg for the presence of EAV-specific antibodies. The results are summarised in table 10.

### Immunisation of the horses

All five horses were immunised by intramuscular (i.m.) injection and intradermal application of the EAV plasmid DNA encoding (parts of) ORF 2, ORF 5, and ORF 7 using a gene gun. The i.m. inoculations were applied to the muscoli semimembranosus/semitendinosus/gluteus. The gene gun application of the DNA was performed on both sides of the neck. The corresponding parts of the skin (40cm<sup>2</sup>) were shaved before to enable a good DNA application using the gene gun. A detailed protocol of the DNA immunisation (including sedation of the horses, amount of DNA, adjuvants, buffers) is provided in example 4. The horses were investigated 24 and 48 h post immunisation for local and systemic reactions. None of the animals developed fever and the local reactions (thickness of the skin, development and involution of papules) are summarised in tables 12 and 13.



### Collection of blood and serum samples

Peripheral blood lymphocytes, serum and plasma of the horses were taken as outlined in Table 12. Up to date, blood and serum samples were taken three times after the 3<sup>rd</sup> booster immunization to measure the kinetics of antibody titres and activities of cytotoxic T-lymphocytes.

### Determination of the maximum cellular <sup>51</sup>Cr-uptake

Titration experiments were performed using (i) constant amounts of <sup>51</sup>Cr with different cell-concentrations and incubation times and (ii) constant incubation times with different cell-concentrations and amounts of <sup>51</sup>Cr.

Target cells ( $5 \times 10^3$ ,  $2 \times 10^4$ , and  $5 \times 10^4$ ) were labeled with 100  $\mu$ Ci for each 90 min., 150 min., and 240 min. The cellular <sup>51</sup>Cr-uptake as well as <sup>51</sup>Cr present in the culture supernatant were determined. In the supernatants of the labeled cultures, a linear increase of <sup>51</sup>Cr was measured (Figure 14a). The amount of cellular <sup>51</sup>Cr also increased with longer incubation times and higher numbers of cells.

Similar results were obtained when these experiments were performed using constant incubation times with different cell concentrations and amounts of <sup>51</sup>Cr (Figure 14a). The subtraction of the <sup>51</sup>Cr present in the cells and the <sup>51</sup>Cr released into the supernatant is shown in (Figure 14b). For the measurement of cytotoxic T-lymphocyte activities in the blood of the immunized horses, it was decided to label  $5 \times 10^4$  target cells with 200  $\mu$ Ci for 240 min.

### Measurement of cytotoxic T-lymphocyte activities after the 2<sup>nd</sup> and 3<sup>rd</sup> booster immunization

The preparation of the target cells including transfection and <sup>51</sup>Cr-labeling was performed as described in detail in Example 3. The isolation of the peripheral blood lymphocytes, the cell culture *in vitro* as well as the restimulation of the effector cells was also performed as outlined in Example 3.

As described above, we labeled  $5 \times 10^4$  target cells with 200  $\mu$ Ci for 240 min. To avoid spontaneous release of <sup>51</sup>Cr by the cells the target cells were kept on ice for 45 min after the first washing step. The effector cells were added to the cultures using effector/target cell ratios of 3:1, 25:1, and 50:1. After incubation for 8 h at 37°C and 5 % CO<sub>2</sub> culture plates were centrifuged at 1000 RPM for 3 min and each supernatant was measured for the presence of <sup>51</sup>Cr in a

scintillation. Negative control culture supernatants consisted of labelled cells without the addition of effector cells (spontaneous  $^{51}\text{Cr}$ -release of the cells). Positive control culture supernatants consisted of cultures of labeled cells after cell lysis using 10 % Triton X-100 (maximum  $^{51}\text{Cr}$ -uptake). The results of the  $^{51}\text{Cr}$ -release of the individual cultures including the effector cells prior to immunization, two weeks after the 2<sup>nd</sup> and two weeks after the 3<sup>rd</sup> booster immunisation (see Table 11) are included as table 18. Figures 15a)- 15e) show the calculated specific lysis  $\geq 0$  according to Hammond SA, Issel CJ, and Montelaro RC (1998): General method for the detection and in vitro expansion of equine cytolytic T lymphocytes. *J Immunol Method* 213: 73-85.

The data sheets containing the  $^{51}\text{Cr}$ -release of the individual cultures (table 18) show the relative small standard deviations between the four individually handled cultures of each sample. Compared with the earlier cytotoxic T-cell assays performed with cells after the first immunization and the 1<sup>st</sup> booster immunization (see example 3), the differences between the negative control culture supernatants (spontaneous  $^{51}\text{Cr}$ -release of the cells) and the positive control culture supernatants (maximum  $^{51}\text{Cr}$ -uptake by the cells) are greater in the current assay. In general, we measured higher percentages of the calculated specific lysis (Fig. 15). In the cultures containing effector cells obtained prior to immunization, however, we also measured relatively high percentages of specific lysis. It would be expected to have higher amounts of  $^{51}\text{Cr}$  released in the supernatants when higher number of effector cells were added to the targets. The values measured in the supernatants of the cultures with effector/target cell ratios of 3:1, 25:1, and 50:1, however, did not show such a consistence in specific lysis. Possibly, the number of antigen expressing target cells (approximately 20 %) are not high enough to enable the measurement of specific lysis. To enlarge the number of antigen positive cells, we used in the current experiments  $5 \times 10^4$  target cells. Other possible reasons for the inconsistency of specific lysis are not efficient restimulations of the effector cells *in vitro* or other unknown methodical details.

#### Summary of the measured cytotoxic T-lymphocyte activities

Due to the inconsistency of the measured specific lysis it is difficult to draw conclusions concerning the antigen(s) most suited for the induction of cytotoxic T-lymphocytes. In an attempt to give an overview of the measured specific lysis we calculated for each ORF the average value (X) of the specific lysis of all different effector/target cell ratios (3:1, 25:1, 50:1) and subtracted

the average value (Y) of the negative controls (results of the cells before immunization), which were calculated similarly.

$$X = \frac{X_1(3:1) + X_2(25:1) + X_3(50:1)}{3} \quad Y = \frac{X_1(3:1) + Y_2(25:1) + Y_3(50:1)}{3}$$

The results show the absolute increase of the cell lysis in percent at the indicated times for each ORF, compared to the values obtained before immunization.

Two different scales were used to express the increase of specific lysis. In example 5, Table 19, measured lysis between 0 and 5 % is given a + (plus). In example 5, Table 20, measured lysis between 0 and 5 % is given a - (minus). Horse Daggy was serologically positive before immunization. This explains the measured specific lysis already two weeks after the first immunization. Horse 'Frieda' had a weak antibody response before the first immunization. Cytotoxic T-lymphocytes directed against the expression product of ORF 2 and 7 were also measured in the blood of this animal two weeks after the first immunization. The serologically negative animals 'Nelke' and 'Friedrich' did not develop cytotoxic T-lymphocytes after the first immunization. Only in horse 'Jessy', also serologically negative before immunization, cytotoxic T-lymphocytes directed against the expression product of ORF 7 were measured. Unfortunately, the specific lysis was not consistent at the following time points. After the 3<sup>rd</sup> booster immunization, however, specific lysis (> 10 %) was measured in all animals. The activity of cytotoxic T-lymphocytes was directed in horse 'Frieda' and 'Jessy' against the expression product of ORF 5, in horse 'Daggy' and 'Friedrich' against the expression product of ORF 7 and in horse 'Nelke' against the expression product of ORF 7.

Table 6: Content of a kit produced for DNA-vaccination of horses using expression vectors harboring and expressing individual gene of equine arteritis virus

Expression vectors	Concentration DNA µg/µl	Total of plasmid DNA mg
pCR3.1-EAV-O2-BX-C5 Gene product: <u>small glycoprotein</u>	1	10
pCR3.1-EAV-O3-BX-C1 Gene product: <u>unknown</u>	1	10
pCR3.1-EAV-O4-BX-C3 Gene product: <u>unknown</u>	1	10
pCR3.1-EAV-O5-BX-C14 Gene product: <u>large envelope glycoprotein</u>	1	10
pCR3.1-EAV-O6-BE-C4 Gene product: <u>membrane protein</u>	1	10
pCR3.1-EAV-O7-BX-C3 Gene product: <u>nucleocapsid protein</u>	1	10
pDP-EAV-O5-BgS-C1 Gene product: <u>large envelope glycoprotein</u>	1	10
pDP-EAV-O7-BgS-C2 Gene product: <u>nucleocapsid protein</u>	1	10
pC3.1-HIS-EAV-O5del-121-C12 Gene product: <u>The N-terminal (121 aa) hydrophilic ectodomain of large envelope glycoprotein</u>	1	10

GD-HD-07-12-1999

Table 7:

Test of EAV specific antibodies in preimmune serum

The results of neutralization test (NT) and ELISA test in which the preimmune sera of 5 horses from Leipzig were screened for existence of a humoral immune response developed during a natural infection with wild type equine arteritis virus (EAV)

Horse	Titer of serum NT <sup>a</sup>	Titer of serum ELISA <sup>a</sup>	Remarks
Daggi	1:40	1:100	positive
Frieda	1:20	1:50	boundary
Friedrich	1:10	<1:50	
Jessy	1:10	<1:50	
Nelke	1:10	<1:50	
Positive Control Serum	1:160 <sup>b</sup> 1:80 <sup>c</sup>	1:200 <sup>c</sup>	positive positive

<sup>a</sup> Multiplicity of infection (MOI): 100 PFU of EAV/1000 RK-13 cells/well

<sup>b</sup> Rabbit antiserum raised against EAV

<sup>c</sup> Horse serum was obtained from Prof. Dr. Ludwig, Berlin

Table 8:

Duration of Immunity:

Determination of antibody titer by neutralization test (NT) in the serum of 5 horses after DNA vaccination with the cDNA EAV ORF 2, 5 and 7 and cDNA encoding equine IL 2.

Horse	Pre- vaccine Serum 22.5.2000 NT-Titer:	1.Post- vaccine Serum 5.6.2000 NT- Titer:	2.Post- vaccine Serum 28.7.2000 NT-Titer:	3.Post- vaccine Serum 22.9.2000 NT-Titer:	4. Post- vaccine Serum 19.1.2001 NT-Titer:	5. Post- vaccine Serum 16.3.2001 NT-Titer:	6. Post- vaccine Serum 11.5.2001 NT-Titer:
Daggi	1:64	1:256	1:256	1:264	1:128	1:96	1:16
Frieda	<1:2	1:256	1:256	1:264	1:64	1:32	1:16
Friedrich	<1:2	1:64	1:128	1:512	1:48	1:32	1:8
Jessy	<1:2	1:256	1:128	1:128	1:16	1:16	1:8
Nelke	1:8	1:256	1:256	1:128	1:16	1:16	1:16

Table 9:

Immunisation schedule with the cDNA EAV ORF 2, 5 and 7 and with cDNA encoding equine IL

2. Combined application of cDNA (in µg) via Gene Gun (GG) and intramuscular injection (i.m.)

Horse	1. Basic immunisation: 23.Mai 2000	1. Booster 6.Juni 2000	2. Booster 21.Juni 2000	3. Booster 14.Juli 2000
Daggy	35 µg GG + 1400 µg i.m	35 µg GG + 1400 µg i.m	35 µg GG + 1400 µg i.m	35 µg GG + 1400 µg i.m
Frieda	35 µg GG + 1400 µg i.m	35 µg GG + 1400 µg i.m	35 µg GG + 1400 µg i.m	35 µg GG + 1400 µg i.m
Friedrich	35 µg GG + 1400 µg i.m	35 µg GG + 1400 µg i.m	35 µg GG + 1400 µg i.m	35 µg GG + 1400 µg i.m
Jessy	35 µg GG + 1400 µg i.m	35 µg GG + 1400 µg i.m	35 µg GG + 1400 µg i.m	35 µg GG + 1400 µg i.m
Nelke	35 µg GG + 1400 µg i.m	35 µg GG + 1400 µg i.m	35 µg GG + 1400 µg i.m	35 µg GG + 1400 µg i.m

Table 10: Age of Horses

horses	age (years)	anti-EAV antibodies*
Daggy	22	++
Frieda	16	?
Jessy	16	-
Friedrich	10	-
Nelke	6	-

\*before immunisation (10/99)

Table 11: Sample collection and immunisation regimen

	DNA application	collection of plasma samples	isolation of PBMC*
prae-immunisation	n.d.	02.03.00	02.03.00
prae-immunisation	n.d.	23.03.00	23.03.00
prae-immunisation	n.d.	22.05.00	22.05.00
Immunisation	23.05.00	05.06.00	05.06.00
1. Booster	06.06.00	20.06.00	20.06.00
2. Booster	21.06.00	13.07.00	13.07.00
3. Booster	14.07.00	28.07.00	28.07.00
	n.d.	25.08.00	25.08.00
	n.d.	22.09.00	22.09.00

\* peripheral blood mononuclear cells

n.d.: not done

Table 12

Table 12  
 Skin Reactions of Horses: 24 hours post injectionem after first immunisation\*

	Daggy	Frieda	Nelke	Friedrich	Jessy
Numbers of papules	le: 6 ri: 6	le: 4 ri: 6	le: 2 ri: 1	le: 5 ri: 5	le: 6 ri: 6
Description of papules	le: severe swollen, epidermis becomes eroded ri: severe swollen, 1 papule without epidermis	le: severe swollen, epidermis becomes severe eroded ri: moderate swollen, Str.c. mild eroded	le: moderate swollen, Str.c. normal ri: see left	le: not to mild swollen, Str.c. normal ri: moderate swollen, Str.c. normal	le: mild to moderate swollen, Str.c. scaled ri: mild swollen, Str.c. scaled
Skin thickness					
i) normal	le: 0.25 cm ri: 0.30 cm	le: 0.30 cm ri: 0.30 cm	le: 0.20 cm ri: 0.20 cm	le: 0.30 cm ri: 0.35 cm	le: 0.40 cm ri: 0.40 cm
ii) Papules	le: 0.60 cm ri: 0.60 cm	le: 0.60 cm ri: 0.60 cm	le: 0.45 cm ri: 0.40 cm	le: 0.40 cm ri: 0.40 cm	le: 0.60 cm ri: 0.60 cm
Diameter	le: 1.1 - 1.2 cm ri: 1.0 cm	le: 0.8 - 1.0 cm ri: 0.7 - 0.8 cm	le: 0.6 - 0.8 cm ri: 0.7 cm	le: 1.0 - 1.1 cm ri: 0.9 - 1.2 cm	le: 0.5 - 0.6 cm ri: 0.5 cm
Features		On both sides: gnath bites with allergic oedema	Very thin skin, le: 2 white circles	1 papule: Str.c. mild swollen, congested and folding of the skin	1 papule with erosion, strong oedema and pruritus

Le: left side of neck; ri: right side of neck; Str.c.: Stratum corneum;  
 \* Immunisation date: 23/05/2000; Skin reaction protocol date: 24/05/2000



Table 13

Table 13  
Skin Reactions of Horses: 48 hours post injectionem after first immunisation\*

	Daggy	Frieda	Nelke	Friedrich	Jessy
Numbers of papules	le: 6 ri: 6	le: 4 ri: 6	le: 2 ri: 1	le: 5 ri: 5	le: 6 ri: 6
Description of papules	le: severe swollen, epidermis becomes eroded ri: severe swollen, 1 papule without epidermis	le: severe swollen, epidermis becomes severe eroded ri: moderate swollen, Str.c. mild eroded	le: moderate swollen, Str.c. normal ri: see left	le: no to mild swollen, Str.c. normal ri: moderate swollen, Str.c. normal	le: mild to moderate swollen, Str.c. scaled ri: mild swollen, Str.c. scaled
Skin thickness					
i) normal	le: 0.25 cm ri: 0.30 cm	le: 0.30 cm ri: 0.30 cm	le: 0.20 cm ri: 0.20 cm	le: 0.30 cm ri: 0.35 cm	le: 0.40 cm ri: 0.40 cm
ii) Papules	le: 0.70 cm ri: 0.60 cm	le: 0.50 - 0.60 cm ri: 0.45 cm	le: 0.45 cm ri: 0.40 cm	le: 0.40 cm ri: 0.40 cm	le: 0.60 cm ri: 0.60 cm
Diameter	le: 1.1 - 1.2 cm ri: 1.0 cm	le: 0.8 - 1.0 cm ri: 0.7 - 0.8 cm	le: 0.6 - 0.8 cm ri: 0.7 cm	le: 1.0 - 1.1 cm ri: 0.9 - 1.2 cm	le: 0.5 ri: 0.9 - 1.2 cm
Features		On both sides: goat bites with allergic oedema	Very thin skin, le: 2 white circles	1 papule: Str.c. mild swollen, congested and folding of the skin	1 papule with erosion, strong oedema and pruritus

Le: left side of neck; ri: right side of neck; Str.c.: Stratum corneum; \* Immunisation date: 23/05/2000; Skin reaction protocol date: 24/05/2000

**Example 3 Cytotoxic T-lymphocyte (CTL Assay)****1. Immunisation of horses****1.1. DNA application and observed skin reactions**

The horses (Daggy, Frieda, Friedrich, Jessy, Nelke) were immunised according to the immunisation regimen (Table 14) by intramuscular (i.m.) injection and intradermal gene gun application of EAV ORF-2, EAV ORF-5, and EAV ORF-7 expression plasmids. The i.m. inoculations were applied to the muscili semimembranosus/semitendinosus/gluteus. The gene gun applications of the DNA were performed on both sides of the neck. To enable an optimal gene gun DNA application the corresponding parts of the skin (40 cm<sup>2</sup>) had been shaved before. The horses were investigated 24 h after the DNA applications for systemic and local reactions. None of the animals developed fever or local reactions such as thickening of the skin, development and involution of papules. Protocols are included as Tables 12 and 13 (see above). Photographs of the skin areas involved were taken 24 h after DNA application.

Table 14. Sample collection and immunisation regimen			
	DNA application	collection of plasma samples	isolation of PBMC*
Prae-immunisation	n.d.	02.03.00	02.03.00
Prae-immunisation	n.d.	23.03.00	23.03.00
Prae-immunisation	n.d.	22.05.00	22.05.00
Immunisation	23.05.00	05.06.00	05.06.00
1. Booster	06.06.00	20.06.00	20.06.00
2. Booster	21.06.00	13.07.00	13.07.00
3. Booster	14.07.00	28.07.00	28.07.00

\*peripheral blood mononuclear cells  
n.d.: not done

**1.2. Detection of EAV-specific antibodies**

Serum and plasma samples of the immunised animals were taken two/three weeks after the DNA applications as outlined in Table 14.

## 2. Preparation of target cells

### 2.1. Transfection experiments in T25 cell culture flasks

Skin fibroblasts ( $1.43 \times 10^6$  cells) were incubated at  $37^\circ\text{C}$  and 5 % and 5 %  $\text{CO}_2$  in T25 ( $25 \text{ cm}^2$ ) cell culture flasks using Dulbecco's modified Eagles medium (DMEM) containing 10 % fetal calf serum (FCS) and antibiotics. Cells were transfected with 100  $\mu\text{g}$  DNA of a Green Fluorescent Protein (GFP) expression plasmid in 1.3 ml Optimem<sup>®</sup> (Gibco) by the addition of 87.5  $\mu\text{l}$  LipofectAMINE<sup>®</sup> (Gibco). After 24 h cells were treated with trypsin and seeded in a concentration of  $5 \times 10^3$  to  $5 \times 10^4$  cells per well in 96-well-plates. About 20 % of the cells were transfected after 24 h of incubation at  $37^\circ\text{C}$  and 5 %  $\text{CO}_2$  as determined by immunofluorescence.

### 2.2. Titration experiments using polyclonal EAV-specific rabbit serum (11.08.98)

Vero-cells ( $10^4$ ) were seeded in each well of a 96-well-plate and grown over night (o/n) in DMEM culture medium containing 10 % FCS and antibiotics at  $37^\circ\text{C}$  and 5 %  $\text{CO}_2$ . The monolayers were subsequently infected with a 1:100 or 1:1000 diluted Vero-cell propagated EAV stock (Institute of Virology, University of Leipzig). The first EAV-specific cytopathic effects (CPE) were observed 36 h post infection and the cultures were immediately fixed for 30 min with ethanol at  $4^\circ\text{C}$ . The 96-well-plate was stored with ethanol at  $-70^\circ\text{C}$ .

For immunohistochemical detection of viral antigens in infected cells, cultures were rinsed for 5 min with phosphate buffered saline (PBS) at room temperature (RT). In order to block endogenous peroxidases, cultures were incubated for 5 min with 7.5 %  $\text{H}_2\text{O}_2$  in methanol. Cells were rinsed with PBS and incubated for 1 h with serum dilutions (polyclonal EAV-specific rabbit serum from 11.08.98, pre-immune [rabbit #98/8] from 10.06.98 ranging from 1:100 and 1:200 up to 1:1600 in 0.05 % Tween 20-PBS. Cultures were rinsed twice with 0.05 % Tween 20-PBS and once with distilled water and incubated for 1 h with a biotinylated anti-rabbit antibody diluted 1:750 at  $37^\circ\text{C}$ . Cells were rinsed as before with Tween 20-PBS and distilled water and incubated for 30 min at  $37^\circ\text{C}$  with streptavidin-peroxidase (1:500 in Tween 20-PBS). Cultures were rinsed again and the substrate AEC (14,25 ml Na-Acetate buffer 0.75  $\mu\text{l}$

ABC, 75  $\mu$ l H<sub>2</sub>O<sub>2</sub> 1:10 diluted in distilled water) was added. Even in the highest antibody dilution (1:1600) clear positive reactions were visible.

### 2.3. Transfection of skin fibroblasts with EAV-specific expression plasmids

Transfection experiments were performed similarly to the protocol described in example 2 using the 24.-well-plate format. Immunohistochemical analysis using the polyclonal EAV-specific rabbit serum (11.08.98), diluted 1:800 in 0.05 % Tween 20-PBS, showed that about 20 % of the cells were transfected with the EAV ORF-2, EAV ORF-5, and EAV ORF-7 expression plasmids.

### 2.4. Culturing of target cells prior to CTL assay

Skin fibroblasts of the horses were cultured in Iscove's modified Eagles medium (IMEM) containing 10 % FCS and antibiotics. Cultures were extended to three T25 cell culture flasks and transfected when being confluent for 80-90 % with each 100  $\mu$ g of the EAV ORF-2, EAV ORF-5, and EAV ORF-7 expression plasmids. After 24 h, cultures were treated with trypsin; cells were counted and labeled in suspension with <sup>51</sup>Cr.

## 3. Preparation of effector cells

### 3.1. Inactivation of EAV using <sup>137</sup>Cs $\gamma$ -rays

In order to obtain viral antigen for the restimulation of EAV-specific effector cells, Vero-cells were infected with EAV at a multiplicity of infection (moi) of 1. After 36 h, cell cultures were freeze/thawed two times and the cultures were centrifuged for 5 min at 1750 x g. The supernatant was pelleted by centrifugation o/n at 19.000 RPM. The pellet was resuspended in 3 ml PBS and the protein concentration was measured using a BCA-Protein Assay<sup>®</sup> (Pierce). Aliquots of the samples containing 2 mg/ml were stored at -70°C.

Samples were exposed on dry ice to <sup>137</sup>Cs  $\gamma$ -rays ranging from 15 Gy up to 1 k Gy. Virus titration on Vero-cells revealed that the infectious titre had decreased only by < log1, even after exposure to 1 k Gy.

### 3.2 Inactivation of EAV using ultraviolet radiation

Because EAV could not be inactivated by  $^{137}\text{Cs}$   $\gamma$ -rays, samples were exposed to ultraviolet radiation (254 nm) at a distance of 5 cm for 5 min up to 80 min. Virus titration on Vero-cells revealed that no infectious virus could be detected after a 5 min exposure. To completely inactivate EAV in the samples used for the restimulation of the effector cells, these samples were exposed for 30 min and stored aliquots containing 2 mg of protein per ml at  $-70^{\circ}\text{C}$ .

### 3.2. Culturing of effector cells prior to CTL assay

PBMC were cultured in IMEM containing 10 % FCS and antibiotics. After stimulation for two days with 2.5  $\mu\text{g/ml}$  pokeweed mitogen, cells were cultured for two days in the presence of 200 U of human recombinant IL-2 (Amersham-Pharmacia). EAV-specific cytotoxic T-cells were restimulated for four days with 30  $\mu\text{g/ml}$  inactivated EAV in the presence of 100 U IL-2. Thereafter, cultures were extended for four days in the presence of 200 U of IL-2. Cells were counted and used in the CTL assay.

## 4. Measurement of EAV-specific cytotoxic T-cells

### 4.1. $^{51}\text{Cr}$ -labeling of target cells

After trypsin treatment, transfected target cells were resuspended in 1.5 ml and labeled for 2 h with 100  $\mu\text{Ci}$   $^{51}\text{Cr}/10^7$  cells. Cells were washed three times with culture medium and  $7.7 \times 10^3$  cells were seeded in each well of a 96 U-bottom cell culture plate in a volume of 150  $\mu\text{l}$ . Cultures were incubated for 4 h at  $37^{\circ}\text{C}$  and 5 %  $\text{CO}_2$  to let cells adhere to the plate.

### 4.2. Addition of effector cells

PBMC were counted and added to the cultures in 100  $\mu\text{l}$  volumes and effector/target cell ratios of 3:1, 25:1, and 50:1. Cells were incubated for 8 h in a total volume of 250  $\mu\text{l}$  at  $37^{\circ}\text{C}$  and 5 %  $\text{CO}_2$ .

#### 4.3. Measurement of $^{51}\text{Cr}$ release in cultures with different effector/target cell ratios

Culture plates were centrifuged at 1000 RPM for 3 min and each supernatant was measured for the presence of  $^{51}\text{Cr}$  in a scintillation counter for 60 sec. Negative control culture supernatants consisted of labeled cells without the addition of effector cells (spontaneous  $^{51}\text{Cr}$  release of the cells). Positive control culture supernatants consisted of cultures of labeled cells after cell lysis using 10 % Triton X-100 (maximum  $^{51}\text{Cr}$  uptake). The results of the  $^{51}\text{Cr}$  release of the individual cultures including the effector cells prior to immunisation, two weeks after the 2nd immunisation, and two weeks after the 3<sup>rd</sup> booster immunisation (Table 14) are included s. table 18. Figures 9a-e) show the calculated specific lysis  $\geq 0$  according to Hammond SA, Issel CJ, and Montelaro RC (1998): General method for the detection and in vitro expansion of equine cytolytic T lymphocytes. *J. Immunol Methods* 213: 73-85.

The data sheets of the  $^{51}\text{Cr}$  release of the individual cultures show a relative small difference between the negative control culture supernatants (spontaneous  $^{51}\text{Cr}$  release of the cells) and the positive control culture supernatants (Maximum  $^{51}\text{Cr}$  uptake by the cells). The specific lysis is therefore difficult to measure, even if the standard deviations between the four individually handled cultures of each sample are small (Table 18).

#### Example 4 DNA-Vaccination of horses

##### 1. Schedule for DNA for Vaccination of horses

May 23, 2000	1 <sup>st</sup> immunization	day 0
June 6, 2000	2 <sup>nd</sup> immunization	day 14
June 21, 2000	3 <sup>rd</sup> immunization	day 29
July 14, 2000	4 <sup>th</sup> immunization	day 51

##### 2. Strategy and preparation of DNA for Vaccination

###### 2.1. Application of DNA using Helios Gene Gun System (BIO-RAD):

###### 2.1.1. Number of shots per animal and per vaccination: 10

###### 2.1.2. Total DNA per shot: 3.5 $\mu\text{g}$

2.1.3. Number of expression vectors used: 7 (the properties and source of the DNA used for vaccination are summarized in Table 15)

- |                                 |                        |
|---------------------------------|------------------------|
| 1. pCR3.1-EAV-O2-BX-C5          | 5. pCR3.1-EAV-O7-BX-C3 |
| 2. pCR3.1-EAV-O5-BX-C14         | 6. pDP-EAV-O7-BgS-C2   |
| 3. pDP-EAV-O5-BgS-C1            | 7. pCR3.1-Horse-IL2    |
| 4. pCR3.1-HIS-EAV-O5del-121-C12 |                        |

2.1.4. DNA of individual expression vector per shot:  $0.5 \mu\text{g} = 500 \text{ ng}$

2.1.5. Total DNA of individual expression vector per animal and per vaccination:  $5 \mu\text{g}$

2.1.6. Total DNA per vaccination per animal:  $7 \times 5 = 35 \mu\text{g}$

2.1.7. Number of animals: 5

2.1.8. Total DNA of individual expression vector per vaccination:  $5 \times 35 = 175 \mu\text{g}$

2.1.9. Number of vaccination: 4

2.1.10. Total DNA of individual expression vector administered:  $175 \times 4 = 700 \mu\text{g}$

## 2.2. Application of DNA through intramuscular route.

2.2.1. Number of inoculations per animal and per vaccination: 4

2.2.2. DNA of individual expression vector per inoculation:  $50 \mu\text{g}$

2.2.3. Total DNA of individual expression vector per animal and per vaccination:  $50 \times 4 = 200 \mu\text{g}$

2.2.4. Number of expression vectors used: 7 (see Table 15)

2.2.5. Total DNA per vaccination per animal:  $7 \times 200 = 1.4 \text{ mg}$

2.2.6. Number of animals: 5

2.2.7. Total DNA of expression vector administered:  $5 \times 1.4 = 7.0 \text{ mg}$

2.2.8. Number of vaccination: 4

2.2.9. Total DNA of individual expression vector administered:  $200 \times 5 \times 4 = 4 \text{ mg}$

2.2.10. Transfection reagent:

DOTAP Liposomal (Roche; Cat.# 1811177)  $50 \mu\text{g/ml}$  BME (3 ml/animal)

Buffer I containing DNA (yellow cup tube)

Lipofectin (Life Technology; Cat.# 18292-011)  $50 \mu\text{g/ml}$  BME (3 ml/animal)

Buffer II (white cup tube) add to DNA prior to application

**Table: 15: Properties of the cDNA used for the undividual DNA-vaccination of horses**

<i>Expression vectors</i>	DNA used per vaccination by $\mu\text{g}/\text{horse}$	
	Gene GUN	Classical i.m.
PCR3.1-EAV-O2-BX-C5 (1 $\mu\text{g}/\mu\text{l}$ )* 1.clone/5. Passage 16.06.1999 Gene product: <u>small glycoprotein</u>	5	200
pCR3.1-EAV-O3-BX-C1 (1 $\mu\text{g}/\mu\text{l}$ )* 1.clone/6. Passage 17.06.1999 Gene product: <u>unknown</u>		
pCR3.1-EAV-O4-BX-C3 (1 $\mu\text{g}/\mu\text{l}$ )* 1.clone/6. Passage 18.06.1999 Gene product: <u>unknown</u>		
pCR3.1-EAV-O5-BX-C14 (1 $\mu\text{g}/\mu\text{l}$ ) 1.clone/5. Passage 05.06.1999	5	200
pDP-EAV-O5-BgS-C1 (1 $\mu\text{g}/\mu\text{l}$ ) 1.clone/5. Passage 07.06.1999	5	200
pCR3.1-HIS-EAV-O5del-121-C12 (1 $\mu\text{g}/\mu\text{l}$ ) 1.clone/6. Passage 04.06.1999 Gene product: <u>large envelope glycoprotein</u>	5	200
pCR3.1-EAV-O6-BE-C4 (1 $\mu\text{g}/\mu\text{l}$ ) 1.clone/5. Passage 15.06.1999 Gene product: <u>membrane protein</u>		
pCR3.1-EAV-O7-BX-C3 (1 $\mu\text{g}/\mu\text{l}$ ) 1.clone/6. Passage 09.06.1999	5	200
pDP-EAV-O7-BgS-C2 (1 $\mu\text{g}/\mu\text{l}$ ) 1.clone/5. Passage 10.06.1999 Gene product: <u>nucleocapsid protein</u>	5	200
pCR3.1-Horse-IL2 (1 $\mu\text{g}/2\mu\text{l}$ ) 2.clone/2. Passage 06.02.2000 Gene product: <u>Horse interleukin-2</u>	5	200



3. Preparation of the DNA for vaccination of horses using  
Helios Gene Gun System (BIO-RAD) and optimization Kit  
(Catalog 165-2424)

3.1. Calculation:

3.1.1. MLQ = 1 (1mg Au/shot)

3.1.2. DRL = 3.5 (3.5 µg DNA/ mg Au, particle = 1.6 µm)

3.1.3. Cartridge length = 13 mm

3.1.4. Nalgene tubing = 750 mm

3.1.5. Factor for Au = 750/13 = 58 mg Au

3.1.6.  $58 \times 3,5 \mu\text{g DNA} = 203 \mu\text{g DNA} \sim 210 \mu\text{g DNA}$

3.2. Procedure:

3.2.1. In a 1.5 ml microfuge tube, weigh out 60 mg Au.

3.2.2. To the measured Au add 210 µl 0.05 M Spermidine.

3.2.3. Vortex # 3.2.2. for few seconds, then sonicate 3-5 seconds.

3.2.4. To the Au and Spermidine mixture, add 210 µg total DNA of seven expression vectors = 30 µg of individual expression vectors).

3.2.5. Mix DNA, Spermidine and Au by vortexing ~ 5 seconds.

3.2.6. Add 210 µl CaCl<sub>2</sub> dropwise into # 3.2.5. while vortexing at a moderate rate

3.2.7. Precipitate for 10 min in room temperature.

3.2.8. Most of the Au will now be in a pellet, but some may be on the sides of the tube.

The supernatant should be relatively clear. Spin the microcarrier solution in

a microfuge for 15 seconds to pellet the Au.

3.2.9. Remove the supernatant and discard.

3.2.10. Resuspend the pellet in the remaining supernatant by vortexing briefly. Wash the pellet three times with 1 ml 100 % ethanol each time.

3.2.11. Spin for 5 seconds in a microfuge between each wash. Discard the supernatants.

3.2.12. After the final ethanol wash, resuspend the pellet in 200 µl of the ethanol solution containing 0.05 mg/ml PVP in ethanol.

3.2.13. Add # 3.2.12 in 2800 µl 0.05 mg/ml PVP in ethanol.

3.2.14. Preparation of cartridges:

May 20, 2000: 300 cartridges were prepared according to the above protocol and the

Helios Gene Gun system Instruction Manual. Each cartridge = single shot contains 3.5 µg DNA corresponding to 0.5 µg = 500 ng DNA of individual expression vectors.

#### 4. Preimmune sera

The preimmune sera of 5 horses (Daggi, Frieda, Friedrich, Jessy, and Nelke) that were obtained in October 1999 and analysed by NT and ELISA tests developed in our laboratory for screening of antibodies against equine arteritis virus (EAV). The results are summarized in Table 16.

Table 16: The results of neutralization (NT) and ELISA tests in which the preimmune sera of 5 horses were screened for existence of a humoral immune response developed during a natural infection with wild type equine arteritis virus (EAV)

No.	Horse	Titre of serum NT <sup>a</sup>	Titre of serum ELISA	Remarks
1	Daggi	1:40	1:100	positive
2	Frieda	1:20	1:50	boundary
3	Friedrich	1:10	<1:50	
4	Jessy	1:10	<1:50	
5	Nelke	1:10	<1:50	
	Positive Control Serum	1:160 <sup>b</sup> 1:80 <sup>c</sup>	1:200	positive positive

<sup>a</sup> Multiplicity of infection (MOI): 100 PFU of EAV/1000 RK-13 cells/well

<sup>b</sup> Rabbit antiserum raised against EAV

<sup>c</sup> Horse serum was obtained from Prof. Dr. Ludwig, Berlin

#### Vaccination protocol

Tuesday, May 23, 2000

1. Race of horses: Jessy: Shetland Pony, Rest: so called "Warm-blooder"
2. Age of horses
  - 2.1. Daggi: 22 years old
  - 3.2. Frieda: 16 years old

3.3. Friedrich: 10 years old

3.4. Jessy: 16 years old

3.5. Nelke: 6 years old

### 3. Vaccination:

3.1. Jessy start 12.15 end 12.22 (10 x shot: 5 per neck side)

3.2. Nelke start 12.30 \* end 12.38 (10 x shot: 5 per neck side)

3.3. Daggi start 12.45 §& end 12.55 (12 x shot: 6 per neck side)

3.4. Frieda start 12.47 §& end 12.57 (12 x shot: 6 per neck side)

3.5. Friedrich start 13.10 § end 13.15 (12 x shot: 6 per neck side)

---

\* Sedativum: Domosedan (Pfizer) = Methyl-4-hydroxybenzoad 1 ml i.v. (10 mg)

§ Rometar (Serum-Werk Bernburg) = Xylazin i.v. Methyl-4-hydroxybenzoad  
& Daggi and Frieda had been vaccinated together

4. Sera: 2<sup>nd</sup> preimmune serum was taken on Monday, May 22, 2000  
(3 ml per animal)

5. May 24, 2000: All vaccinated horses developed skin reactions "DTH-reaction" at the target of the shot.

Tuesday, June 6, 2000

6. Sera: 1<sup>st</sup> post vaccinated serum was taken on Monday, June 5, 2000 (3 ml per animal)

### 7. Vaccination:

3.1. Frieda start 12.25\* end 12.30 (10 x shot: 5 per neck side)

3.2. Daggi start 12.27\* end 12.33 (10 x shot: 5 per neck side)

3.3. Jessy start 12.37\* end 12.43 (10 x shot: 5 per neck side)

3.4. Nelke start 12.44\* end 12.47 (10 x shot: 5 per neck side)

3.5. Friedrich start 12.49\* end 12.54 (10 x shot: 5 per neck side)

---

\* Sedativum: Rometar = Xylazin i.v. Methyl-4 hydroxybenzoad 2%

(Serum-Werk Bernburg)

## Vaccination protocol

Tuesday, June 21, 2000

8. Sera: 2<sup>nd</sup> post vaccinated serum was taken on Monday, June 20, 2000 (1.5 ml per animal)

## 9. Vaccination:

3.1. Frieda	start	12.10*	end	12.16	(12 x shot: 6 per neck side)
3.2. Daggi	start	12.17*	end	12.20	(12 x shot: 6 per neck side)
3.3. Jessy	start	12.25*	end	12.28	(12 x shot: 6 per neck side)
3.4. Nelke	start	12.29*	end	12.32	(11 x shot: 5 & 6 per neck side)
3.5. Friedrich	start	12.36*	end	12.39	(12 x shot: 6 per neck side)

\* Sedativum: Rometar = Xylazin i.v. Methyl-4 hydrooxybenzoad 2%

(Serum-Werk Bernburg)

## Vaccination protocol

Friday, July 14, 2000

10. Sera: 3<sup>rd</sup> post vaccinated serum was taken on July 13, 2000 , 1.5 ml per animal

## 11. Vaccination:

3.1. Daggi	start	12.00*	end	12.07	(12 x shot: 6 per neck side, 530 psi)
3.2. Frieda	start	12.08*	end	12.11	(12 x shot: 6 per neck side, 530 psi)
3.3. Jessy	start	12.21*	end	12.24	(12 x shot: 6 per neck side, 430 psi)
3.4. Nelke	start	12.25*	end	12.27	(12 x shot: 6 per neck side, 500 psi)
3.5. Friedrich	start	12.35*	end	12.42	(12 x shot: 6 per neck side, 400 psi)

\* Sedativum: Domosedan (Pfizer) = Methyl-4-hydrooxybenzoad 1 ml i.v. (10 mg)

§ Rometar (Serum-Werk Bernburg) = Xylazin i.v. Methyl-4-hydrooxybenzoad

12. Sera: 4<sup>th</sup> post vaccinated serum was taken on July 28, 2000 , 1.5 ml per animal

## 13. Determination of neutralizing antibody

The determination of neutralizing antibodies of individual horse sera (30 serum samples that was labelled with code number 1-30, see Table 3) was performed. The results of neutralization tests are summarized in Table 17.

Table 17: The results of neutralization tests (NT) in which the sera of five horses were screened for detection of a humoral immune response developed after DNA vaccination with the cDNA of ORFs 2, 5, and 7 of equine arteritis virus (EAV) as indicated in Table 15 and 16

Horse	NT-Titre of individual horses taken at the different times prior and post DNA-Vaccination <sup>a</sup>					
	Preimm serum 1 20.10.1	preimm serum 2 22.05.2	post vaccinate serum 1 05.06.20	post vaccinate serum 2 20.06.20	post vaccinate serum 3 13.07.20	post vaccinated serum 4 28.07.2000
1. Daggi	1:16 (#01)	1:64 (#06)	1:256 (#11) 1:256 <sup>e</sup>	1:256 (#16)	1:256 (#21) 1:256 <sup>e</sup>	1:256 (#26)
2. Frieda	<1:2 (#02)	<1:2 (#07)	1:256 (#12) 1:256 <sup>e</sup>	1:256 (#17) 1:256 <sup>e</sup>	1:128 (#22)	1:256 (#27) 1:256 <sup>e</sup>
2. Friedrich	<1:2 (#03)	<1:2 (#08)	1:64 (#13)	1:128 (#18)	1:128 (#23)	1:128 (#28)
4. Jessy	<1:2 (#04)	<1:2 (#09)	1:256 (#14) 1:256 <sup>e</sup>	1:256 (#19) 1:256 <sup>e</sup>	1:256 (#24) 1:256 <sup>e</sup>	1:128 (#29)
5. Nelke	<1:2 (#05)	1:8 (#10)	1:256 (#15) 1:512 (#15)	1:128 (#20)	1:256 (#25) 1:512 <sup>e</sup>	<1:2 <sup>c</sup> (#30) 1:256 <sup>d</sup>

<sup>a</sup> DNA-Vaccination of horses was performed as follows:

May 23, 2000: 1<sup>st</sup> immunization (day 0), June 6, 2000: 2<sup>nd</sup> immunization (day 14), June 21, 2000: 3<sup>rd</sup> immunization (day 29), and July 14, 2000: 4<sup>th</sup> immunization (day 51)

<sup>b</sup> The number in parenthesis indicate the code number of individual sera used by neutralization test, that was performed by Dr. Herzog, Veterinary laboratory at the Institute for clinical analysis, 71611 Ludwigsburg, Germany.

<sup>c</sup> This titre has to be checked again.

<sup>d</sup> The titre 1:2 was a type mistake. The correct titre was found to be 1:256, (Dr. Herzog laboratory, 08/20/2000)

<sup>e</sup> The results of the second analysis obtained from those sera with a titre of 1:256, (Dr. Herzog laboratory, 09/06/2000)

**Biological material**

14. Sera: 5<sup>th</sup> post vaccinated serum was taken on August 25, 2000 ( 2 x 1,5 ml per animal)

15. Sera: 6<sup>th</sup> post vaccinated serum was taken on September 22, 2000 (2 x 1,5 ml per animal)

16. Sera: 7<sup>th</sup> post vaccinated serum was taken on October 23, 2000 (2 x 1,5 ml per animal).

**Example 5**  
**Table 18:**

### <sup>51</sup>Cr-release of the individual cultures

Dose	Cells	ORP	Negative Positive			Prior to Immunization			After the 2 <sup>nd</sup> Booster			After the 3 <sup>rd</sup> Booster						
			Control	Control	Control	3 to 1	25 to 1	50 to 1	3 to 1	25 to 1	50 to 1	3 to 1	25 to 1	50 to 1				
1	Transfected	2	Mean	781	448	821	710	503	444	512	538	584	E.T	3 to 1	25 to 1	50 to 1		
				815	485	881	528	473	812	417	450	849						
				858	549	930	509	580	481	402	688	541						
				552	542	538	457	769	518	407	415	738						
				854	808.5	532.5	551	548.25	486.25	384.5	628.75	858						
				70,000/77	48,071/821	112,374/453	110,188/561	109,355/683	418,753/878	104,018/74	159,638/72	804/901						
				Specif. Lysis	0.28704/885	0.34537/131	0.38351/884	0.37389/854	0.25534/944	0.10447/804	0.34028/884	0.84588/189					0.87182/868	
				5	211	468												
				252	504													
				243	638													
2	Transfected	7	Mean	250	255	287	102	259	283	228	233	E.T	3 to 1	25 to 1	50 to 1			
				277	277	930	228	258	259	232	280					247		
				285	288	888	223	284	258	242	278					252		
				225	282	288	244	274	301	200	223					247		
				281.75	277.5	288.25	221.75	270.75	280.25	229	243.75					248,888/57		
				30,888/891	17,818/886	44,319/880	21,781/888	11,828/884	22,371/818	17,825/728	21,519/885					2,888/75133		
				Specif. Lysis	0.08281/534	0.11787/855	0.18515/518	0.16287/833	0.09388/332	0.12879/881	0.08233/805					0.12462/72	0.12389/81	
				350	812	448	485	483	407	271	240					251		
				308	584	553	383	489	459	288	286					350		
				281	808	448	433	830	591	241	283					484		
3	Transfected	7	Mean	270	877	435	385	388	430	301	287	483	E.T	3 to 1	25 to 1	50 to 1		
				257.25	883.75	470.5	417.5	512.5	416.75	277.25	278.25	394.5						
				40,888/768	64,450/847	55,314/857	43,781/111	48,325/488	84,573/884	21,823/528	27.5	22,247/728					115,888/888	
				Specif. Lysis	0.01828/812	0.55181/885	0.36383/188	0.30285/712	0.68488/2	0.34827/940	-0.08383/838	-0.07007/885					0.38946/888	

**Table 18 a)**

2. Cyt-Assay, 10/11.08.2000

[illegible]

	Transfected	%
225	308	271
267	248	289
228	404	281
242	334	244
107	353	267
217	289	257
210	250	247
218	268	258
220	268	257
224	300.5	257.5
20.4893089	54.2728402	287.25
Mean		
SD		
Spearman's rho		

Transfected	7	507	470	507
		590	483	
		584	480	
		708	433	
		530	408	
		571	517	
		585	501	
		582	777	
Mean		739	554.625	
SD		148.892314		
Specif. Lysis				

**Table 18 b)**



2. CTL-Assay, 10./11.08.2000

[illegible]

**Table 18 c)**

2. CTL-Assay, 10/11.08.2000

[illegible]

**Table 18 d)**

2. CTL-Assay, 10/11.08.2000

[illegible]

**Table 18 e)**

Table 19: Summary of the measured cytotoxic T-lymphocyte activities

horse	orf	immunization	1.booster	2.booster	3.booster
Frieda	2	++	++	-	+
	5	+	++	-	+++
	7	++	+	+	-
Daggy	2	+++	-	-	++++
	5	+++	++	-	-
	7	+++	-	-	-
Nelke	2	+	-	-	-
	5	+	+	-	-
	7	-	-	-	+++
Friedrich	2	-	-	+	+++
	5	+	+	+	-
	7	+	+	-	-
Jessy	2	+	+	-	-
	5	-	-	++++	+++
	7	++	+++	-	-

$$x = \frac{x_1(3:1) + x_2(25:1) + x_3(50:1)}{3} = \text{after immunization}$$

$$y = \frac{y_1(3:1) + y_2(25:1) + y_3(50:1)}{3} = \text{before immunization}$$

$x - y$  = absolute increase of the specific lysis in percent

**x:**

- - = 0%
- + = >0-5%
- ++ = >5-10%
- +++ = >10-20%
- ++++ = >20-30%

Table 19

Table 20: Summary of the measured cytotoxic T-lymphocyte activities

horse	orf	immunization	1.booster	2.booster	3.booster
Frieda	2	+	+	-	-
	5	-	+	-	++
	7	+	-	-	-
Daggy	2	++	-	-	+++
	5	++	+	-	-
	7	++	-	-	-
Nelke	2	-	-	-	-
	5	-	-	-	-
	7	-	-	-	++
Friedrich	2	-	-	-	++
	5	-	-	-	-
	7	-	-	-	-
Jessy	2	-	-	-	-
	5	-	-	+++	++
	7	+	++	-	-

$$x = \frac{x_1(3:1) + x_2(25:1) + x_3(50:1)}{3} = \text{after immunization}$$

$$y = \frac{y_1(3:1) + y_2(25:1) + y_3(50:1)}{3} = \text{before immunization}$$

$x - y$  = absolute increase of the specific lysis in percent

x:

- - = 0-5%
- + = >5-10%
- ++ = >10-20%
- +++ = >20-30%

Table 20

## References

- Balasuriya, U. B. R., Hedges, J. F., Nadler, S. A., McCollum, W.H., Timoney, P. J. and MacLachlan, N. J. (1999) Genetic stability of equine arteritis virus during horizontal and vertical transmission in an outbreak of equine viral arteritis. *J. Gen. Virol.* **80**, 1949-1958.
- Balasuriya, U. B. R., MacLachlan N. J., DeVries, A. A. F., Rossitto, P. V., and Rottier, P. J. M. (1995). Identification of a neutralization site in the major envelope glycoprotein (G<sub>L</sub>) of equine arteritis virus. *Virology* **207**, 518-527.
- Balasuriya, U.B., Patton, J.F., Rossitto, P.V., Timoney, P.J., McCollum, W.H., and MacLachlan, N.J.(1997). Neutralization determinants of laboratory strains and field isolates of equine arteritis virus: identification of four neutralization sites in the amino-terminal ectodomain of the G(L) envelope glycoprotein. *Virology* **232**, 114-128.
- Barry M.A. and Johnston S.A (1997). Biological features of genetic immunization. *Vaccine* **15**, 788-795.
- Bradford, M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of proteins utilizing the principle of protein dye binding. *Anal. Biochem.* **72**, 248-254.
- den Boon, J.A., Snijder, E.J., Chirnside, E.D., de Vries, A.A., Horzinek, M.C., and Spaan, W.J.(1991). Equine arteritis virus is not a togavirus but belongs to the coronaviruslike superfamily. *J Virol* **65**, 2910-2920.
- Glišin, V., Crkvenjakov, R., and Byus, C. (1974). Ribonucleic acid isolated by cesium chloride centrifugation. *Biochemistry* **13**, 2633-2637.
- Hedges, J.F., Balasuriya, U.B.R., Timoney, P.J., McCollum, W.H., and McLachlan, N.J. (1999). Genetic divergence with emergence of novel phenotypic variants of equine arteritis virus during persistent infection of stallions. *J Virol* **73**, 3672-3681.

- Pasquini, S., Xiang, Z., Wang, Y., He, Z., Deng, H., Blaszczyk-Thurin, M., and Ertl, H.C. (1997) Cytokines and costimulatory molecules as genetic adjuvants. *Immunol. Cell Biol.* 75, 397-401.
- Patton, J.F., Balasuriya, U.B.R., Hedges, J.F., Schweidler, T.M., Hullinger, P.J., and MacLachlan, N.J. (1999). Phylogentic characterization of a highly attenuated strain of equine arteritis virus from the semen of a persistently infected standardbred stallion. *Arch Virol* 144, 817-827.
- Pedersen, K.W., Van Der Meer, Y. Roos, N., and Snijder, E. (1999). Open reading frame 1a-encoded subunit of the arterivirus replicase induce endoplasmic reticulum-derived double-membrane vesicles which carry the viral replication complex. *J Virol* 73, 2016-2026.
- Pirzadeh, B., and Dea, S. (1998). Immune response in pigs vaccinated with plasmid DNA encoding ORF5 of porcine reproductive and respiratory syndrome virus. *J. Gen. Virol.* 79, 989-999.
- Pycck, J.F. (1998). Equine viral arteritis risk from imported semen. *The Veterinary Record*, 26, 699.
- Rösen-Wolff, A., Ben-Hur, T., Becker, Y., and Darai, G. (1988). Comparative analysis of the transcripts mapped in the BamHI DNA fragment B of avirulent HSV-1 HFEM, virulent HSV-1 F, and their intratypic recombinant viruses. *Virus Res.* 10, 315-324.
- Rösen-Wolff, A., Ben-Hur, T., Becker, Y., and Darai, G. (1988). Comparative analysis of the transcripts mapped in the BamHI DNA fragment B of avirulent HSV-1 HFEM, virulent HSV-1 F, and their intratypic recombinant viruses. *Virus Res.* 10, 315-324.
- Rösen-Wolff, A., Scholz, J. and Darai, G. (1989) Organotropism of latent herpes simplex virus type 1 is correlated to the presence of a 1.5 kb RNA transcript mapped within the BamHI DNA fragment B (0.738 to 0.809 map units). *Virus Res.* 12, 43-52.

Rösen-Wolff, A. and Darai, G. (1991) Identification and mapping of the UL56 gene transcript of herpes simplex virus type 1. *Virus Res.* 19, 115-126.

Stadejek, T., Björklund, H., Ros Bascunana, C., Ciabatti, I.M., Scicluna, M.T., Amaddeo, D., McCollum, W.H., Autorino, G.L., Timoney, P.J. Paton, D. J., Klingeborn, B., and Belak, S. (1999). Genetic diversity of equine arteritis virus. *J. Gen. Virol.* 80, 691-699.

Snijder, E., Van Tol, H., Pedersen, K.W., Raamsman, M.J.B., and De Vries, A.A.F. (1999). Identification of a novel structural protein of arteriviruses. *J Virol* 73, 6335-6345.

Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F., and Higgins, D. G. (1997). The CLUSTAL\_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res.* 25, 4876-4882.

van der Meer, Y., Van Tol, H. Locker J.K., and Snijder, E. (1998). DORF1a-encoded replicase subunits are involved in the membrane association of the arterivirus replication complex. *Journal of Virology* 72, 6689-6698.

van Dinten, L.C., Rensen, S., Gorbalenya, A. E. and Snijder, E.J. (1999). Proteolytic processing of the open reading frame 1b-encoded part of arterivirus replicase is mediated by nsp4 Serine protease and is essential for virus replication. *J Virol* 73, 2027-2037.

Will, H., Cattaneo, R., Koch, H.G., Darai, G., Schaller, H., Schellekens H., van Eerd, P.M.C.A., and Deinhardt, F. (1982). Cloned HBV DNA causes hepatitis in a chimpanzee *Nature* 299, 740-742.

Will, H., Cattaneo, R., Darai, G., Deinhardt, F., Schellekens H., and Schaller, H. (1985). Infectious hepatitis B virus from cloned DNA of known nucleotide sequence, *Proc. Natn. Acad. Sci. U.S.A.* 82, 891-895.



**Claims**

1. Vaccine composition which is protective against equine arterivirus (EAV) infections in horses and induces a cellular immune response, comprising a open reading frame nucleic acid (ORF) 2, ORF 5 and/or ORF7 of EAV.
2. Vaccine composition according to claim 1, wherein said vaccine composition comprises ORF 2, ORF 5 and ORF7 of EAV.
3. Vaccine composition according to claim 1 or 2, wherein said vaccine composition further comprises one or several ORF(s) selected from the group of ORF 1a, ORF 1b, ORF 3, ORF 4, ORF 6.
4. Vaccine composition according to any one of claims 1 to 3, wherein said nucleic acid is cDNA.
5. Vaccine composition according to any one of claims 1 to 4, wherein said vaccine composition comprises one or several nucleic acid vectors each comprising said ORF or ORFs.
6. Vaccine composition according to claim 5, wherein said vector(s) is/are expression vector(s).
7. Vaccine composition according to claim 6, wherein said expression vector(s) comprise(s) a eukaryotic cis-acting transcription/translation sequence functionally linked to said ORF(s).
8. Vaccine composition according to claim 7, wherein said expression vector is selected from the group of pCR3.1, pcDNA3.1/His A, pcDNA3.1/His B, pcDNA3.1/His C, and pDisplay (pD).
9. Vaccine composition according to any one of claims 1 to 8, further comprising the nucleic acid encoding equine interleukin 2 (IL-2) or a vector or expression vector comprising said nucleic acid encoding IL-2.
10. Vaccine composition according to any one of claims 1 to 9, further comprising pharmaceutically acceptable carrier or excipient.
11. Vaccine composition according to any one of claims 1 to 10, further comprising one or several adjuvants selected from the group of Muramyl Dipeptide (MDP), Montanide 720, Poly Inosine:Cytosine (Poly I:C) or plasmid DNA comprising unmethylated cytosine, guanine dinucleotide sequence motifs (CpG).
12. Vaccine composition according to any one of claims 1 to 11, consisting of expression vectors comprising ORF2, ORF5 and ORF7 of EAV, respectively, and optionally carrier,

excipients or adjuvants and an expression vector comprising the nucleic acid encoding IL-2.

13. Vaccine composition according to claim 12, wherein ORF 2 is SEQ ID No. 2, ORF 5 is SEQ ID No. 5 or SEQ ID No. 9 and ORF 7 is SEQ ID No. 7.
14. Vaccine composition according to any one of claims 1 to 13, wherein the nucleic acid or nucleic acid vector or expression vector is encapsulated into cationic liposomes.
15. Nucleic acid vector comprising nucleic acid selected from the group of ORF 1a, ORF 1b, ORF 2, ORF 3, ORF 4, ORF 5, ORF 6 and/or ORF 7 of EAV.
16. Nucleic acid vector according to claim 15, wherein said nucleic acid is DNA.
17. Nucleic acid vector according to claim 15 or 16, wherein said nucleic acid vector is an expression vector.
18. Nucleic acid vector according to any one of claims 15 to 17, wherein said expression vector comprises a eukaryotic cis-acting transcription/translation sequence functionally linked to said nucleic acid(s) specific for said ORF(s).
19. Nucleic acid vector according to any one of claims 15 to 18, wherein said expression vector is selected from the group of pCR3.1, pcDNA3.1/His A, pcDNA3.1/His B, pcDNA3.1/His C, and pDisplay (pD).
20. Nucleic acid vector according to any one of claims 15 to 19, wherein said nucleic acid vector comprises a nucleic acid selected from the group of SEQ ID No. 2, SEQ ID No. 5, SEQ ID No. 9 and/or SEQ ID No. 7.
21. A method for prophylaxis or treatment of EAV infection in a horse, comprising
  - (i) coating one or several nucleic acid vector(s) according to any one of claims 15 to 20 onto carrier particles;
  - (ii) accelerating the coated carrier particles into epidermal cells of the horse *in vivo*; and
  - (iii) inducing a protective or therapeutic immune response in said horse upon or after exposure to EAV; and
  - (iv) monitoring the reduction of EAV-associated symptoms or the reduction of horizontal or vertical transmission.
22. The method according to claim 21, wherein the carrier particles are gold.
23. A method for prophylaxis or treatment of EAV infection in a horse, comprising
  - (i) injecting a vaccine composition according to claim 1 to 14 or one or several nucleic acid vector(s) according to any one of claims 15 to 20 into muscular cells of the horse *in vivo*; and
  - (ii) inducing a protective or therapeutic immune response in said horse upon or after

exposure to EAV, and

(iii) monitoring the reduction of EAV-associated symptoms or the reduction of horizontal or vertical transmission.

24. Use of one or several nucleic acid vector(s) according to any one of claims 15 to 20 in the manufacture of a vaccine for the prophylaxis and treatment of EAV infections.

**Summary**

The invention belongs to the field of animal health and in equine arterivirus (EAV). The invention provides vaccine compositions comprising open reading frame (ORF) 2, ORF 5 and ORF 7 nucleic acid of EAV, and vectors comprising said ORFs. The invention further relates to the use of said ORFs and vectors in the manufacture of a medicament for the prevention and treatment of EAV infections.

## SEQUENCE LISTING

&lt;110&gt; Boehringer Ingelheim Vetmedica GmbH

&lt;120&gt; Equine Arterivirus Vaccine

&lt;130&gt; 1-1281-I

&lt;140&gt; not yet assigned

&lt;141&gt; 2002-01-19

&lt;160&gt; 61

&lt;170&gt; PatentIn Ver. 2.1

&lt;210&gt; 1

&lt;211&gt; 9031

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

<223> Description of Artificial Sequence: ORF 1a and b of  
RAV

&lt;400&gt; 1

taaaactttt	gccacctgac	cgccgtgagg	ctgggtttgag	gttggtattac	aaccattaac	60
gcgaacaaag	gaccgggttg	ctgtctaaaa	qaggacttcg	cttatggctt	ggagacctgg	120
gtttgggcat	caatgogago	tctggagggc	tgaatttcca	oattatgagg	ggttcgctc	180
agcgagcttg	goatatcaca	acacgcagct	gcaagctgaa	gagotactac	gtttgtgaca	240
tctctgaagc	agactgggtc	tgtttgcctg	ctggcaacta	cggcggttac	aatccaccag	300
gggacggagc	ttgcgggttac	aggtgcttgg	ccttcattgaa	tggcgccact	gttggtgctgg	360
ctgggttcag	ttctgacttg	tgggtgtgatg	atgagttggc	ttatogagtc	tttoaattgt	420
caccacagct	cacgggttacc	atccacaggtg	ggcgagtttg	tccgaatgco	aagtacgcaa	480
tgattttgtga	caagcagcac	tggcgcgctga	aacgtgcaaa	gggcgctcggc	ctgtgtctcg	540
atgaaagctg	tttcaggggc	atctgcaatt	gccaacgcac	gagtggaaca	ccacotgca	600
ccgtgtcagc	cgccgtgtta	gatcacatac	tggaggcggc	gacgtttggc	aacgttcgcg	660
tggttacacc	tgaagggcag	ccacgcctcg	taccagcgcc	gcgagttcgt	cccagcgcoa	720
actctctctg	agatgtcaaa	gatccggcgc	ccgttccgcc	agtaaccaaa	ccaaggacca	780
agcttgccac	accgaaccca	actcaggcgc	ccatccagc	accggcgacg	cgacttcaag	840
gggcctcaac	acaggagcca	ctggcgagtg	caggagttgc	ttctgactcg	gcacctaaat	900
ggcggtgtgg	caaaactgtg	tacagctccg	cggagcgctt	tccgaccgaa	ctgggtacaac	960
gtgctcggtc	cgttggggac	gttcttgttc	aagcgctacc	gctcaaaaac	ccagcagtcg	1020
agcggtatag	catgactctg	aagatgatgc	gttcaogctt	cagttggcac	tgcgacgtgt	1080
ggtacocctt	ggctgtaate	gcttgtttgc	tccctatatg	gccatctott	gctttgctcc	1140
ttagcttttc	cattgggttg	atacccagtc	tgggcaataa	tggtgtttctg	acagcgcttc	1200
tgggtttcaco	agctaattat	gttgcgctcaa	tggacatca	atgtgaagggt	gcggcttgct	1260
tagccttgct	ggaagaagaa	cactattata	gagcggctcg	ttggcgcccg	attacaggcg	1320
cgctgtcgct	tgtgctcaat	ttactggggc	aggtaggota	tgtagctcgt	tccacctttg	1380
atgcagctta	tgttcccttg	actgtgttcg	atctttgcag	ctttgctatt	ctgtacctct	1440
gcgcgaatcg	ttgctgggag	tgttcgggac	gctgtgtgcg	agttgggoot	gccacgcag	1500
ttttgggctc	cacggggcaa	cgagtttcca	aactggcgct	cattgatttg	tgtgaccact	1560
tttcaaagcc	caccatcgat	gttgtgggca	tggcaactgg	tgggagcgga	tgttacacag	1620
gaacgcgcgc	aatggagcgt	cagtggtgct	ctacggtgga	ccotcaactcg	ttogaccaga	1680
agaaggcagg	agcgactgtt	tacctcacc	ccctgtcaa	cagcggtgca	gcgctgcagt	1740
gcctcaatgt	catgtggaag	cgaccaattg	ggtccactgt	ccttggggaa	caaacaggag	1800
ctgttgtgac	ggcggtcaag	agtatctott	tctcacctcc	ctgotgcgtc	tctaccactt	1860
tgoocacccg	accgggtgtg	accgttgtcg	accatgctct	ttacaaccgg	ttgactgctt	1920
cagggggtoga	tcccgcttta	ttgcgtgttg	ggcaagggtga	ttttctaaaa	cttaatccgg	1980
ggttcgggct	gataggtgga	tggattctatg	ggatatgcta	ttttgtgttg	gtgggttgtgt	2040
daaottttba	ctgottacot	atcaaatgtg	gcattggcac	cgcgcaccct	ttctgccgca	2100
gagtggtttc	tgtaccgcgc	accaagaccc	aagagcactg	ccatgctgga	atgtgtgcta	2160
gcgctgaagg	catctctctg	gactctctcg	gtttaaactca	gttacaaagt	tactggatcg	2220
cagcogtcac	tagcggatta	gtgatcttgt	tgggtctgca	ccgcctggcc	atcagcgctt	2280

tggacttgtt	gactotagot	tcccttttag	tgttgcctgt	gttcccttgg	gcactctgtg	2340
ggcttttact	tgtttgcagt	ctcgctgggt	ctgctgtgaa	aatacagttg	ttggcgacgc	2400
tttttgtgaa	tctgtttctt	ccccaaagct	cccttgtcac	tatgggatac	tgggcgtgcg	2460
tggcggtttt	ggocgtttac	agtttgatgg	gcttgcaggt	gaaagtgaat	gtgcccaggt	2520
gtgtgacacc	tgcccatttt	ctgctgtgtg	cgaggtcagc	tggacagtca	agagagcaga	2580
tgtccgggtt	cagcgtgtgt	gccccacca	attcactgct	tggagtggtt	cgtgatttgt	2640
atgtcacagg	cacaactcgg	ctgtacatac	ccaaggaagg	cgggatgggt	tttgaagggo	2700
tattcaggto	aocgaaggcg	cgcggcaacg	tgggttcgt	ggctggtagc	agctacggca	2760
cagggtcagt	gtggaocagg	aacaacgagg	tctgtctact	gacagcgtca	cacgtgggtg	2820
gocgcgctaa	catggccact	ctgaagatcg	gtgacgcaat	gotgactctg	actttcaaaa	2880
agaatggcga	ottogccgag	goagtgacga	cacagtccga	gctcccaggg	aattggccac	2940
agttgcattt	cgcccaacca	acaaccgggc	ccgcttcagt	gtgcaactgc	acaggagatg	3000
aagaaggctt	gctcagtggc	gaggtttgtc	tggcgtggac	tactagtggc	gactctggat	3060
ctgcagtggg	tcagggtgac	gctgtggtag	gggtccacac	cggttcgaac	acaagtgggt	3120
ttgctcagaa	gaccacccca	agcggaaaac	tccttggcgc	cgacaccgtg	actttgtcat	3180
cactgtcaaa	gcattttcaa	ggccctttga	catcaatccc	gaaggacatc	cctgacacaa	3240
ttattgcgca	tgttgatgct	gttccctcgt	ctctggccat	gctgattgat	ggcttatcca	3300
atagagagag	cagcctttct	ggacctcagt	tgttggtaat	tgtttgtttt	atgtgggtct	3360
atcttaacca	acctgcttac	ttgoccttat	tgttgggctt	ctttgccgct	aactttctcc	3420
tgcocaaaaa	tgttggccgc	cctgtgggtc	ctgggcttct	atgggttgtg	tgcctcttca	3480
caccgctttc	catgcgcttg	tgtttgttcc	atctgggtct	tgtaccgtc	acgggaaacg	3540
tgatatcttt	gtgggtctac	atcactgccc	ctggcacgtc	ttacctttct	gagatgtggt	3600
tcggaggcta	tcccaccatg	ttgtttgtgc	cacggttccc	agtgtaccag	ttccccggt	3660
gggtctattg	cacagactta	ggcgtatgca	gcctaccat	gotggctgct	gccctcggtc	3720
acaccctgtt	adtggatgtg	ttctccgct	caggtcgtt	tgacaggact	ttcatgatga	3780
aatacttctt	ggaggaggga	gtgaaagaga	gtgtcacogo	ctcagtcacc	cgcgottatg	3840
gocaaaccaat	taccacggag	agtctcactg	caacatttag	tgcctcact	gatgatgact	3900
tcdaatttct	ctctgatgtg	cttgactgtc	gggcgctccg	atcggcaatg	aactctgcgt	3960
ccgctctcac	aagtttttcaa	gtggcgagct	atugtaacat	ccttaatgca	tccttgcaag	4020
tcgactcgtg	cgtctgtcgt	agtcgcagag	taatggcaaa	actggctgat	tttgccgttg	4080
aaacaagaag	aacagctgga	gaccgtgttg	tggttatcga	cggctcggac	cgcattggct	4140
aottcaaaag	cgaatttggtg	ctgggttctt	tgacacacaa	agtagtaggc	gggtcttaggt	4200
gocacatttg	tgacgtcgtt	aaggaaagaag	ccaatgacac	ccaggttaag	ccaatgcccc	4260
gcaggagacg	cgcgaagggg	ctgcctaaag	gtgotcagtt	ggagtgggac	cgtcacccagg	4320
agagagaagag	gaacgcgggt	gatgatgatt	ttgcggtctc	gaatgattat	gtcaagagag	4380
tgcocaaagta	ctgggatccc	agcgacaccc	gaggcacgac	agtgaataat	gcgggoacta	4440
cctatocagaa	agtgggtgac	tattcaggca	atgtgcatta	cgtggagcat	cagggaagat	4500
tgotagacta	cgtgctgggg	aaggggagct	atgaaggcct	agatcaggac	aaagtgttgg	4560
acctcacaaa	catgcttaaa	gtggacccca	cggagctctc	ctcraaagac	aaagocaaag	4620
cgcgtcagct	tgtctcatctg	ctgttggatc	tggctaaacc	agttgaggca	gtgaatcagt	4680
taaatctgaga	gcgcgccaca	tctttcccg	cgatgtgggg	cgtcggacct	ttgtgactc	4740
taagagacaag	ggtttctgtg	ctctacacag	tcgcacaaatg	tttttagctg	cccggaactt	4800
tttattttaac	atcaaatattg	tgtgcgacga	agagttcaca	aagaccccaa	aagacacact	4860
gcttgggtac	gtacggcct	gccctggtta	ctggtttatt	ttccgtogta	cgcacccgtc	4920
gctgattgat	goataotggg	acagtatgga	gtgcgtttac	gcgcttccca	ccatatctga	4980
ttttgatgtg	agcccagggtg	acgtcgcgag	caggggcgag	cgatgggatt	ttgaatctcc	5040
cggaggaggc	cgtgcacaaa	gtctcacagc	tgatctgggt	caagcttttc	aagggttcca	5100
cggagcctct	tattcctatg	atgacaagggt	ggcagctgot	gtcagtgggt	accggtatcg	5160
gtcggacggc	gtcttgtata	acaccggttg	gggcaacatt	ccatattctg	tcccaaccaa	5220
tgttttggaa	gccacagctt	gctaccgtgc	tggatgtgag	gocgttaocg	acgggaocaa	5280
cgtcatcgca	acnaattgggc	ccttcccgga	gcaacaaccc	ataccggaca	toccaaagag	5340
cgtgcttgac	aactgcgctg	acatcagctg	tgaogcttct	atagcgoccg	ctgcagagac	5400
agccctgtgt	ggagattttag	agaaatacaa	ccatccacg	cagggttttg	tgttcgctag	5460
tgttttctcc	atgggtgcggg	cgtacttaaa	agaggagatt	ggagacgctc	caccactota	5520
cttgcactct	actgtacct	ctaaaaatc	acaagccgga	attaaocggc	ctgagtttcc	5580
tacaaagtct	ttacagagct	actgtttgat	tgatgacatg	gtgtcacagt	ccatgaaaag	5640
caatctacaa	accgccacca	tggcgacttg	taaacggcga	tactgttcca	aatacaagat	5700
taggagcatt	ctgggcacca	acaattacat	tggccttaggt	ttgcgtgcct	gcctttcggg	5760
ggttacggoc	gcattccaaa	aagctggaaa	ggatgggtca	ccgatttatt	tgggcaagtc	5820
aaaattcgac	ccgataccag	ctcctgacaa	gtactgcctt	gaaacagaco	tggagagttg	5880
tgatcgctcc	accccggtt	tgggtgcgtg	gttgcctact	aattottatt	ttgagcttag	5940
tggccagccc	gagttgggtg	acagctacgt	gttgaaattg	tgtcacgata	tagtrcgtgc	6000
gggtagtgtg	gcattccaca	aaocgggggg	tttgtcatct	ggagacccta	tcaattccat	6060

```

ttccaatacc atctattcat tgggtgotgta caccacagcag atgttgctat gtggacttga 6120
aggctatttc ccagagattg cagaaaaata tcttgatggc agcctggagc tgcgggacat 6180
gttcaagtac gttcagatgt acatctactc ggacgatgtg gttctaacca caccacaacca 6240
gcattacgag gccagctttg accgctgggt ccccccacctg caggcgctgc taggtttcaa 6300
ggttgaccca aagaaaaactg tgaacaccag ctccctcttc tttttgggct gccgggtcaa 6360
gcaagtggac ggcaagtgtt atotagocag tcttcaggac cgcgttacac gctctctgtt 6420
ataccacatt ggtgcaaaaga atccctcaga gtactatgaa gotgctgttt ccatotttaa 6480
ggactccatt atctgctgtg atgaagactg gtggacggac ctccatcgac gtatcagtgg 6540
cgctgcgcgt accgacggag ttgagttccc caccattgaa atgttaacat ccttccgcac 6600
caagcagtat gagagtgcg tgtgcacagt ttgtgggggc gcccccgtgg ccaagtctgc 6660
ttgtggaggg tgggtctgtg gcaattgtgt cccgtaccac gcgggtcatt gtcacacaa 6720
ctogctcttc gccaactgcg ggcacgacat catgtacgc tcoacttact gcacaatgtg 6780
tgagggttcc ccaaaacaga tggtaacaaa agtgcctcac ccgatcctgg atcatttgtc 6840
gtgccacatt gattacggca gtaaagagga actaaotctg gtagtggcgg atggtcgaac 6900
aadatcacog cccgggcgct acaaagtggg tcacaaggta gtcgcctggg ttgcagatgt 6960
gggagggaac attgtgtttg ggtgcgggtcc tggatcacac atcgcagtac cacttcagga 7020
tacgctcaag ggcgtggtgg tgaataaagc tctgaagaa gccgcgcct ctgagtacgt 7080
ggaagggaac cctgggagtg ggaagactct tcacctgggtc aaagatgtgc tagccgtggt 7140
cggtagcgcg acctgggttc tgcccacoda cgcgtccatg ctggactgca tcaacaagct 7200
caaacaagcg ggcgcgcat catactttgt ggtgcccag tatcacagtt ttgactttcc 7260
ccggootggc agtggaacaa tcacagtgcg actgcoacag gtcggaacda gtgaggga 7320
aacctttgtg gatgaggtgg cctacttctc accagtggtat ctggcgcgca ttttaaccca 7380
gggtcgagtc aagggttacg gtgatttaaa tcagctcggg tgcgtcggac ccgcgagcgt 7440
gccacgtaac ctttggctcc gacattttgt cagcctggag cccttgcgag tgtgccatcg 7500
attcggcgct gctgtgtgtg atttgatcaa gggcatttat ccttattatg agccagctcc 7560
acataccact aaagtgggtg ttgtgccaaa tccagacttt gagaaagggtg tagtcatcac 7620
ogootaacac aaagatcgcg gtcttgggtc aogcaoaatt gatcaattc aaggctgtac 7680
attccctggt gtgactcttc gactgcgcac accccaatca ctgacgcgc ccgogocagt 7740
tgtggcggtt actaggggtg otcagggaatt atacatctac gacccctttg atcagcttag 7800
cgggttggtt aagttoacca aggaagcaga ggcgcaggac ttgatccatg gcccacctac 7860
agcatgocac ctggggccaag aaattgacct ttggtccaat gagggcctcg aatattacaa 7920
ggaagtcaac ctgctgtaca cacacgtccc catoaaggat ggtgtaatac acagttaccc 7980
taattgtggc cctgcctgtg gotgggaaaa gcaatccaac aaaatttctg gcctcccag 8040
agtggcacaa aatttgggtc accactatto ccagactta ccaggatttt gccccatacc 8100
aaaagaactc gotgagcatt ggoccgtagt gtccaatgat agataocoga attgcttga 8160
aattacctta cagcaagtat gtgaactcag taaaccgtgc tcagcgggct atatggttg 8220
acaattctgt ttctgtcaga cgcctggtgt gacatcttac tggcttactg aatgggtcga 8280
cggcaaaagcg cgtgctctac cagattcctt attctogtcc ggtagggttc agactaacag 8340
ccgcgctttc ctcgatgaag ccgaggaaaa gtttgccgc gctcaccctc atgootgttt 8400
gggagaaatt aataagtcca cctggggagg atcccacttc atcttttccc aatatttaoc 8460
accattgcta cccgcagacg ctgttgccct ggtagggtgt tcatgggtg ggaagctgc 8520
taaagotgct tgcagcgttg ttgatgtcta tgotcoatca tttgaacctt atotacacc 8580
tgagacactg agtcgcgtgt acaagatbat gatcgatttc aagcctgtga ggcttatgg 8640
gtggagaaac gcgacctttt atgtocaaga ggtgttgat gcagttacat cagcactagg 8700
agotgtgtcc aaactcatca aagtgcggc caatgagcct gtttcattcc atgtggcatc 8760
agggtagcaga adcaacgcgc tggtagcgcc ccaggotaaa atttcaattg gaggctacgc 8820
cgccgagtg gcaotgtcaa ctgaaccgoc acctgctggg tatgcatcg tgcggcgata 8880
tattgtaaag aggtccctca gotcaacaga agtgttcttg tgocgcaggg gtgttgtgt 8940
ttccacctca gtgcagacca tttgtgcact agagggatgt aaactctgt tcaacttctt 9000
acaaattggg tcagtcattg ggccogtgtg a 9031

```

&lt;210&gt; 2

&lt;211&gt; 684

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; Description of Artificial Sequence:ORF2 of RAV

&lt;400&gt; 2

```

tgctattttct gtgattgatg cagcgctttt cttttctcatg ctactttgcat tggctgttgt 60
tactgtgttt cttttctggc tcaattgttg catcgccgc agcttgggtg cgcgggtgttc 120

```

```

acgagggtgcg cggttacagac ctgtttaagg atttgacgtg cgacaacctg cgcgcgaaag 180
atgccttccc gagtctggga tatgtctgt cgattggcga gtcgaggcta tcgtatatgc 240
tgcaggattg gttgcttgc gcgcaccgca aggaagttat gccttccaat atcatgccta 300
tgcccggtct tactcctgat tgctttgacc atotggagtc ttctagctat gctccattta 360
tcaatgccta tgggcaggca attttgagtc aatacccaca agagctccag ctcgaagcca 420
tcaactgtaa attgcttgc gtggttgcac cggcattgta tcataattac catotagcca 480
atttgaccgg accggccaca tgggtcgtgc ctacagtggg ccagttgcac tattatgctt 540
cttctcttat ttttgcttca tctgtggaag tgttggcagc aataatacta ctatttgcac 600
gcataccact agtgacacga gtgtacatct cttttaocgc gctaattgta ccttcccgtc 660
gcacttccag cggcactttg ccgc

```

&lt;210&gt; 3

&lt;211&gt; 501

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; Description of Artificial Sequence: ORF 3 of EAV

&lt;400&gt; 3

```

acgggcccaca tgggtcgtgc ctacagtggg ccagttgcac tattatgctt cttctcttat 60
ttttgcttca tctgtggaag tgttggcagc aataatacta ctatttgcac gcataccact 120
agtgcacacga gtgtacatct cttttaocgc gctaattgta ccttcccgtc gcacttccag 180
cggcactttg cgcgcggcga agattttgta gtgcacacgg gttatgaata tgcgggggtc 240
actatgttag tgcacttggt tgccaacttg gttctgacat ttccgagctt agttaattgt 300
tcccgccctg tgaatgtctt tgctaattgt tcttgcgtgc aagtgggttg tagtcatacc 360
aaactaacta ctgggttggg tcaactttct ttttcccttg tagatgaaga tctacggctg 420
catatcaggg ctactdttat ttgttggttt gccttggttg tgggtgcactt tctacccatg 480
ccacgctgca gaggtcgtg a

```

&lt;210&gt; 4

&lt;211&gt; 458

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; Description of Artificial Sequence: ORF4 of EAV

&lt;400&gt; 4

```

tgaagatcta cggctgcata tcaggcctac tcttatttgt tggtttgctt tgttggttgg 60
gcactttcta cccatgccac gctgcagagg ctctgaattt tacttacatt agtcattggat 120
tgggccaagt gcacggtcac gaggggtgta ggaattttat taatgtcact cattctgcac 180
ttctttatct taatcccacc actcccactg cgcgggctat aactcattgt ttacttctgg 240
ttctggcagc caaaatggaa caaccoaacg ctactatctg gctgcagctg cagccgtttg 300
ggtatcatgt ggctggcgat gtcattgtca acttgggaaga ggacaagagg catccttact 360
ttaaactttt gagagcgccg gctttaccgc ttggttttgt ggctatagtt tatgttcttt 420
tacgactggt acgttgggtt caaogatggt atctatga

```

&lt;210&gt; 5

&lt;211&gt; 767

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; Description of Artificial Sequence: Description of Artificial Sequence ORF 5 of EAV

&lt;400&gt; 5

```

tacgttggggc tcaacgatgt tatctatgat tgtattgcta ttcttgcctt ggggtgcgcc 60
atcacatgct tactttctcat actacaocgc tcagcgcttc acagacttca ccttgtgtat 120

```



```

gctgacggat cgcggcggtta ttgccaattt gctgcgatat gatgagcaca ctgcttttga 180
caattgttcc gccagtaaaa cotgttggta ttgcacattc ctggacgaac agattatcac 240
gttttgaacc gattgtgatg acacotacgc ggtccpagtt gctgaggtec tggaacaggg 300
gcatggacag tacagtgcgc tgtttgatga catgcccact tttatttact atggccgtga 360
attcggcata gttgtgttgg atgtgtttat gttctatccc gtttttagtcc tgtttttctt 420
atcagtacta ccttatgcta cgtttattct tgaattgtgt gtatottatto tgtttataat 480
ctatggcatt tacagcgggg cctacttggc catgggoata tttgoggcca cgtttgctat 540
acattcaatt gtggtcctcc gccattact gtggttatgc ctggccttggc gataccgctg 600
tacgottcac gcgtccctta tatcagctga ggggaaagtg taccocgtag accccggact 660
cccggttgcc gccgtgggca atcggttgtt agtcccaggt agggccacta tggattatgc 720
agtggcctac ggcagcaaa g tcaacctgtt gaggttgggg gcagctg 767

```

&lt;210&gt; 6

&lt;211&gt; 489

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; Description of Artificial Sequence:ORF 6 of EAV

&lt;400&gt; 6

```

atgggagcca tagattcatt ttgtggtgac gggatttttag gtgagtatct agattacttt 60
attctgtccg tcccaactctt gctgttgcct actagggtatg tagcactctgg gttagtgtat 120
gttttgaactg oottgttcta ttcttttga tttagcagctt atattttgggt tgttatagtt 180
ggaagagcct tttctactgc ttatgctttt gtgcttttgg ctgcttttct gttattagta 240
atgaggatga ttgtgggtat gatgcctcgt cttcgggtcca ttttcaacca tcgccaactg 300
gtggttagctg attttgttga cacacctagt ggacctgttc ccatcccccg ctcaactact 360
caggtagtgg ttgogggcaa cgggtacacc gcagtbggtg acaagcttgt cgatggogtc 420
aagacgatca cgtccgcagg ccgcctcttt tcgaaacgga cggcggogac agcctacaag 480
ctacaatga 489

```

&lt;210&gt; 7

&lt;211&gt; 333

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; Description of Artificial Sequence:ORF7 of EAV

&lt;400&gt; 7

```

tggtaacaag cttgtcgatg gogtcaagac gatcaogtcc gcaggccgcc tcttttcgaa 60
acggacggcg gcgacagcct acaagctaca atgaactact gcgoatgttc ggtcagatgc 120
gggtccgcga accgcocgcg caaccactc aggtattat tgcagagcct ggagacctta 180
ggcatgattt aaatcaacag gagcgogcca ccttttcgtc gaacgtacaa cggttcttca 240
tgattgggoa tggttcactc actgcagatg cgggaggact caagtacacc gtcagtgggg 300
ttcctacoaa acaaatccag cgcaaagttg ogc 333

```

&lt;210&gt; 8

&lt;211&gt; 495

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

<223> Description of Artificial Sequence:Partial ORF1  
Sequence

&lt;400&gt; 8

```

tggcaacctt ctccgctact ggatttggag ggagttttgt tagggactgg tccctggact 60
taccgcagcg ttgtgagcat ggcgcgggat tgtgtgcga agtggacggg tccacottat 120
gcgcagagtg ttttcgggt tgcgaaggaa tggagcaatg tccctgggtt ttcattgggac 180

```

```

tggttaaaact gggttcgcca gtccagtggt gacataagtt cctgattggg tggatcagag 240
ctgccaaagt caccgggggt tacaatttcc ttgagctgtt gcaaoaccct gctttcgccc 300
agctgogtgt gggtgatgct aggttagcca ttgaagaggc aagtgtgttt atttccactg 360
accacgcgtc tgctaagcgt tccctggcg ctagatttgc gctgacaccg gtgtatgcta 420
acgcttgggt tgtgagcccg gctgctaaca gtttgatagt gaccactgac caggaaacaag 480
atgggttctg ctgggt

```

&lt;210&gt; 9

&lt;211&gt; 363

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

<223> Description of Artificial Sequence: Truncated ORF5  
of EAV

&lt;400&gt; 9

```

tacgttgggc tcaacgatgt tatctatgat tgbattgcta ttcttgcttt ggggtgcgcc 60
atcacatgct tacttctcat actacacccg tcagcggttc acagaattca ccttggtgat 120
gctgacggat cgcggcgctta ttgccaattt gctgcgatat gatgagcaca ctgctttgta 180
caattgttcc gccagtaaaa cotgttggta ttgcacattc otggacgaac agattatcac 240
gtttggaacc gatttgtgatg acacctacgc ggtcccagtt gctgaggtcc tgggaacaggc 300
gcctggaccg tacagtgcgc tgtttgatga catgccccct ttpatttaot atggccgtga 360
att

```

&lt;210&gt; 10

&lt;211&gt; 12704

&lt;212&gt; DNA

&lt;213&gt; Equine arteritis virus

&lt;400&gt; 10

```

gctcgaagtg tgtatgggtg catatacggc toaccaccat atacactgca agaattacta 60
ttcttgtggg cccctctcgg taaatcctag agggcttccc tctcggtatt gcgagattcg 120
tcgttagata acggcaagtt ccccttctta ctatccctat tctatcttgt ggcttgacgg 180
gtcactgcca tcgtcgtcga tctctatcaa ctacccttgc gactatggca acctctcccg 240
ctaactggatt tggaggaggt tttgttaggg actggtccct ggacttacct gacgcttgtg 300
agcatggcgc gggatttgtg tgcgaagtgg aoggtccac cttatgogcc gagtgttttc 360
gcgggttgcga aggaatggag caatgtcctg gcttgttcat gggactgtta aaactgggtt 420
cgccagttcc agtgggacat aagtccctga ttggttggta tgcagctgcc aaagtccacc 480
ggcggttaca tttccttgag ctgttgcaac accctgcttt cggccagctg cgtgtgggtg 540
atgctagggt agocattgaa gaggaagtgt tgtttatttc cactgacacg gcgtctgcta 600
agcggtttccc tggcgctaga tttgcgctga caccgggtga tgctaacgct tgggttgtga 660
gcccggctgc taacagtttg atagtgaaca ctgaccagga acaagatggg tctgtctggt 720
taaaactttt gccaacctgac cgccgtgagg ctggtttgcg gttgtattac aaccattacc 780
gcgaacaaag gaccgggtgg ctgtctaaaa caggacttcg attatggctt ggagacctgg 840
gtttgggcat caatgcgagc tctggagggg tgaaattcca cattatgagg ggttcgcctc 900
agcgagcttg gcatatcaca acacgcagct gcaagctgaa gagctactac gtttgtgaca 960
tctctgaagc agactgggtc tgtttgcctg ctggcacaota cggcggttac aatccaccag 1020
gggacgggagc ttgcgggttac aggtgcttgg ccttcatgaa tggcgccact gtttgtctgg 1080
ctggttgcag tttctgaottg tgggtgtgat atgagttggc ttatcgagtc tttcaattgt 1140
caccacggtt caoggttacc atcccagggt ggcgagtttg tccgaatgco aagtacgcaa 1200
tgatttgtga caagcagcac tggcgcgtoa aacgtgcaaa gggcgctggc ctgtgtctcg 1260
atgaaagctg tttcaggggc atctgcaatt gccaacgcac gagtggaaca ccacctgca 1320
ccgtgtcagc cgcggtgcta gatcacatac tggaggcggg gacgtttggc aacgttcgca 1380
tggttacacc tgaaggggcag ccacgcctcg taccagcgcc gcgagttcgt cccagcgcca 1440
actcttctgg agatgtcaaa gatccggcgc cgttcccgcc agtaccaaaa ccaaggacca 1500
agcttggcac accgaaccca actcaggcgc ccatcccgag acccgcgacg cgaactcaag 1560
gggcctcaac acaggagcca ctggcgagtg caggagttgc ttctgaactg gcacctaaat 1620
ggcggtgtgg caaaaactgtg tacagctccg cggagcgctt tgggacgaa ctggtacaac 1680
gtgctcggtc cgttggggac gttcttgttc aagcgctacc gctcaaaaac ccagcagtg 1740
agcgggtatac catgactctg aagatgatgc gttcacgctt cagttggcac tgcgacgtgt 1800

```

ggtaacccttt ggctgtaate gcttgttttgc tccctatatg gccatctctt gctttgtctc 1860  
ttagcttttgc cattgggttg ataccoagtg tgggcaataa tgttgttctg acagcgcttc 1920  
tgggtttcagc agptaattat gttgcgtcaa tggaaocatca atgtgaaggc gcggcttgct 1980  
tagcctttgct ggaagaagaa cactattata gagcggtccg ttggcgcccg attacaggcg 2040  
cgctgtcgct tgtgetcaat ttactggggc aggtaggcta ttagctcgt tccacctttg 2100  
atgcagctta tgttcccttgc actgtgttcg atcttttgcag ctttgcctatt ctgtacctct 2160  
gocgcaatcg ttgctggaga tgcctcggac gotgtgtgcg agttgggccc gccadgcctg 2220  
ttttgggctc caocgggcaa cgagtttcca aactggcgct cattgatttg tgtgaccact 2280  
tttcaaagcc caccatcgat gttgtgggca tggcaactgg ttggagcgga tgttacacag 2340  
gaaccgcccgc aatggagcgt cagtggtgct ctacgggtgga ccctcactcg ttcgaccaga 2400  
agaaggcagg agcgactgtt tacctcacc cccctgtcaa cagcggttca gcgctgcagt 2460  
gocctcaatgt catgtggaag cgaccaattg ggtccactgt ccttggggaa caaacaggag 2520  
ctgcttgtgac ggcggtcaag agtatctctt tccacctcc ctgctgcgtc totaccactt 2580  
tgccaccctg acccggtgtg accgttgtcg accatgctct ttacaacggg ttgactgctt 2640  
caggggtcga tcccgtctta ttgctgttg ggaaagggtga ttttctaaaa cttaatccgg 2700  
gggtccggct gatagggtgga tggatttatg ggatatgcta ttttgbgttg gtgggttgtt 2760  
caactttttac ctgcttacct atcaaagtgt gcatttggac ccgagacct ttctgcccga 2820  
gagtgctttc tgtaccctgc accaagaccc aagagcactg ccctgctgga atgtgtgcta 2880  
gcgctgaagg catctctctg gactctctgg ggttaactca gttacaaagt tactggatcg 2940  
cagccgctcac tagcggatta gtgactttgt tggcttgcca ccgcttggcc atcagcgctt 3000  
tggacttctt gactctagct tccccttag tgttcttctg gttcccttgg gcactctgtt 3060  
ggcttttact tgcctgcagt ctgctgggtg ctgctgtgaa aatacagttg ttggcgagcg 3120  
tttttctgaa tctgttctt ccccaagcta ccttctgca tatgggatac tggcgctgcg 3180  
tggcggtttt ggccgtttac agtttctgag gcttgcgagt gaaagtgaat gtgcccctgt 3240  
gtgtgacacg tgcccatctt ctgctgctgg cgaggtcagc tggacagtca agagagcaga 3300  
tgctccgggt cagcgctgtt gccccacca attcactgct tggagtggtt cgtgatgtt 3360  
atgtcacagg cacaactcgg ctgtacatac ccaaggaaag cgggatgggt tttgaagggc 3420  
tattcaggct accgaaggcg cgggcaacg tcggcttctg ggctggtagc agctacggca 3480  
oagggtcagt gtggaccagg aacaacgagg tctgctact gacagcgtca oacttggctg 3540  
gcggcgctaa catggccact ctgaagatcg tgcagcact gctgactctg actttcaaaa 3600  
agaatggcga ctctgcgag gcagtgaaga cactgtccga gctcccaggc aattggccac 3660  
agttgcattt cgcaccaacca acaacggggc ccgcttcatg gtgactgct acaggagatg 3720  
aagaaggctt gctcagtggt cagggtttgtc tggcggtggc tactagtggc gactctggat 3780  
ctgagtggtt cagggtgac gctgtggtag ggggtccacac cgggttcgaac acaagtgggt 3840  
tctgactaagt gacaccccca agcggaaaaa tccctggcgc cgacaccgtg actttgtcat 3900  
cactgtcaaa gcattctcaca ggccctttga catcaatccc gaaggacatc cctgacaaca 3960  
ttattgocga tgttgatgct gttccctcgtt ctctggcact gctgattgat ggcttatcca 4020  
atagagagag cagcctttct ggacctcagt tgttgttaat tgttgtttt atgtgggtct 4080  
atcttaacca acctgcttac ttgoccttat tgotgggctt ctttggcgct aactctctcc 4140  
tgccaaaaag tgttggccgc cotgtggtoa otgggottct atggctgtgc tgcctcttca 4200  
caocgctttc catgcgcttg tgcctgttcc atctggtctg tgcctaccgt acgggaaacg 4260  
tgatatcttt gtgggtctac atoactgocg ctggcacgtc ttacctttct gagatgtggt 4320  
tcggaggtca tccacccatg ttgtttgtgc caggttctct agtgaccag ttccccggct 4380  
gggtctattg gcagctacta ggggtatgca gcactaccat gctggctgct gccctcggtc 4440  
acacctgtt actggatgtg ttctccgct cagggtogct tgacaggact ttcagtatga 4500  
aatacttctt gggaggaggga gtgaaagaga gtgtcacccg ctccagtcacc cgcgcttatg 4560  
gcaaaccaat taccaggag agtotoactg caacattagc tgccctcact gatgatgact 4620  
tccaattcct ctctgatgtg ctctgactgt gggcgctcog atcggaatg aatctgcgtg 4680  
ocgctctcac aagttttcaa gtggcgagc atcgtaacat ccttaatgca tcttgcgaag 4740  
tcgatogtga cgtgtcgt agtcgcagac taatggcaaa actggctgat tttgcggtt 4800  
aacaagaagt aacagctgga gacogtgttg tggttatoga cggctctggc cgcctggctc 4860  
acttcaaga cgatttgggt ctgggttctt tgaccaccaa agtagtaggc ggttctaggt 4920  
gcaccatttg tgacgtcgtt aaggaaagag ccaatgacac ccaggttaag ccaatgcaca 4980  
goaggagacg ccgcaagggc ctgcctaaag gtgctcagtt ggaagtggga cgtcaccagg 5040  
aaggagaagag gaacgcgggt gatgatgat ttgcggtoto gaatgattat gtoaagagag 5100  
tgccaaaagta ctgggatccc agcgacaccc gaggoaogac agtgaaaatc gccggcacta 5160  
cctatcagaa agtggttgac tattcaggca atgtgcatta cgtggagcat caggagatc 5220  
tgctagacta cgtgctgggc aaggggagct atgaaggcct agatcaggac aaagtgttg 5280  
acctcacaac catgctraaa gtggacccca cggagctctc ctocaaagac aaagccaagg 5340  
cgcgtcagct tgcctcatct ctgttgatc tggctgaccc agttgaggca gtgaatcagt 5400  
taaaotgaga gcgcgccaca tcttcccgg cagctgggg cgccggacct ttgctgactc 5460  
taaaagacaag ggtttcgttg ctctacacag tgcacaaatg tttttagctg cccgggactt 5520  
tttatttaac atcaaatttg tgtgcgacga agagttcaca aagaccccaa aagacacact 5580

gcttgggtac gtacgcgcct gccctgggta ctgggtttatt ttccgtcgta cgcaccgggc 5640  
 gctgattgat goatactggg acagtatgga gtgogtttac gcgcttccca ccatatctga 5700  
 ttttgatgtg agccacgggt agctgcaggt gacgggagag ccatgggatt ttgaatctcc 5760  
 cggaggaggc cgtgcaaaac gtctcacagc tgatctgggt caogcttttc aagggttoca 5820  
 cggagocctct tattcctatg atgacaaggt ggcagctgct gtoagtgggt acccgatctg 5880  
 gtcggacggc gtcttgtata acacocgttg gggcaaoatt ccattattctg tcccaacca 5940  
 tgctttggaa gacacagctt gctaccgtgc tggatgtgag gccgttaccg accgggacca 6000  
 cgtcatcgca acaattgggc doctcccgga gcaacaacco ataccggaca tcccaagag 6060  
 cgtgcttgac aactgogctg acatcagctg tgacgcttcc atagcgcccg ctgcagagac 6120  
 agccctgtgt ggagatttag agaaatacaa cctatccacg cagggttttg tgttgcctag 6180  
 tgtttttotcc atgggtgoggg cgtacttaaa agaggagatt ggagacgctc caccactcta 6240  
 cttgocatot actgtaccat ctaaaaatcc acaagccgga attaacggcg ctgagtttcc 6300  
 taaaaagtct ttaacagagct actgtttgat tgatgacatg gtgtcacagt ccatgaaaag 6360  
 caatctacaa accgccaoca tggcgacttg taaacggcaa taotgttcca aatacaagat 6420  
 taggagcatt ctgggaccca acaattacat tggccttaggt ttgctgtgct gcctttcggg 6480  
 ggttaogggc gcattccaaa aagctggaaa ggatgggtca ccgatttatt tgggcaagtc 6540  
 aaaattcgac ccgataccag ctccctgacaa gtaactgctt gaaacagacc tggagagttg 6600  
 tgatcgctcc acccgggctt tgggtgogttg gtctgctact aatcttattt ttgagctagc 6660  
 tggccagccc gagtgggtgc acagctacgt gttgaattgc tgtcacgata tagttgtggc 6720  
 gggtagtgta goattcaoca aacggggggg tttgtcactt ggagacccta tcaacttcoat 6780  
 ttccaatacc atctattcat tgggtgctgta caccacgac atgttgcctat gtggacttga 6840  
 aggtatbttc ccagagattg acatctaotc ggaogatgtg gttctaaoca caccacaacca 6900  
 gttcaagtac gttcgagtg taccgtgggt cccccctg caggcgctgc taggtttcaa 7020  
 goattacggc gcoagctttg tgaacaccag ctccccctcc tttttgggct gccggttcaa 7080  
 ggttgaccca aagaaaactg atctagccag tottcaggac cgcgttacac gctctctgtt 7140  
 gcaagtggac ggcaagtgtt atccctcaga gtactatgaa gctgctgttt ccactcttta 7200  
 ataccacatt ggtgcaaaag atgaagactg gtggagcgag ctccatcgac gtatcagttg 7260  
 ggactccatt acctgctgtg acogaogggg ttgagttccc caocattgaa atgttaacat ccttccgcac 7320  
 cgtgocgct gagagtgccg tgtgcacagt ttgtggggcc gcccccgtgg ocaagttctgc 7380  
 caagcagtat tgggtctctg gcaattgtgt cactgaccac gcgggtcatt gtcacacaac 7440  
 ttgtggaggg gccaactgog ggcaogacat catgtacogc tcaactactt goacaatgtg 7500  
 ctgctctctc ocaaaacaga tggtaocaaa agtgctctac ccgactctgg atcatttggc 7560  
 tgaggggttcc gattacggga gtaagaggga actaactotg gtagtggcgg atggctgaac 7620  
 gtgocacatt cccggggcgt acaagtggtt tgcacaaggt gtcgcccgtg ttgcagatgt 7680  
 aacatcaccg gggaggcaac attgtgtttg ggtgcggtcc tggatcacac atcgacgtac cacttcagga 7740  
 tagctcaag ggcgtgggtg tgaataaagc tctgaagaac gccgcccgtt ctgagtagct 7800  
 ggaaggaccc cctgggagtg ggaagacttt tcactgggtc aaagatgtgc tagccgtggt 7860  
 cggtagcgcg accttgggtg tgcccaccca cgcgtccatg ctggactgca tcaacaagct 7920  
 caaacagagc ggcggccgat catactttgt ggtgcccagg tatacagttc ttgactttcc 7980  
 ccggcctggc agtggaacaa tcacagtgcg aotgccacag gtcggaacca gtgagggaga 8040  
 aacctttgtg gatgaggtgg octactttct accagtggat ctggcgcgca ttttaaccca 8100  
 gggctcagtc aagggttacg gtgatttaaa tcagctgggg tgctcggac ccgcgagcgt 8160  
 gccacgtaac ctttggctcc gacattttgt cagcctggag cccttgcgag tgtgoccatg 8220  
 attcggcgct gctgtgtgtg atttgatcaa gggcatttat octattatg agccagctcc 8280  
 acataccact aaagtgggtg ttgtgcaaaa tocagaactt gagaaagggt tagtcatcac 8340  
 cgcctaccac aaagatcgcg gtcttgggtc ccgcacaatt gattcaattc aaggctgtac 8400  
 attccctgtt gtgactcttc gactgcccac accccaatca ctgacggcgc cggcgcgagt 8460  
 tgtgggggtt actagggcgt ctcaaggaatt atacatctac gacccctttg atcagcttag 8520  
 cgggttgttg aagtccaoca aggaagcaga ggcgcaggac ttgatccatg gccoacotac 8580  
 agcatgccac ctggggccaag aaattgacct ttggtocaat gagggoctcg aatattacaa 8640  
 ggaagtcaac ctgctgtaca cacacgtccc catcaaggat ggtgtaatac acagttaccc 8700  
 taattgtggc cctgcctgtg gctgggaaaa gcaatccaac aaaatttctg gcctcccag 8760  
 agtggcaca aatttgggct accactatcc ccagactta ccaggatttt gccocatacc 8820  
 aaaagaactc gctgagcatt ggcccgtagt gtccaatgat agataccoga attgcttgca 8880  
 aattacctta cagcaagtat gtgaactcag taaaccgtgc tcagcgggct atatgggtgg 8940  
 acaatctgtt ttogtgocga cgcctgggtg gacatcttac tggcttaotg aatgggtoga 9000  
 cggcaaaagc cgtgctctac cagattccct attctcgtcc ggtaggttct agactaacag 9060  
 ccgctcttcc ctcgatgaag ccgaggaaaa gtttgccgct gctcacctc atgocgtgtt 9120  
 gggagaaatt aataagtcca ccgtgggagg atcccactc atcttttccc aatatttacc 9180  
 accattgcta cccgcagacg ctggtgccct ggtaggtgct tcattggctg ggaagctgc 9240  
 taaagctgct tgcagcgttg ttgatgtcta tgcctcataa tttgaacct atctacacc 9300  
 tgagacactg agtcgcgtgt acaagattat gategatttc aagccgtgta ggcttatggt 9360

```

gtggagaaac ggcacctttt atgtccaaga ggggtgttgat gcagttacat cagcactago 9420
agctgtgtcc aaactcatca aagtgcgggc caatgagoot gtttcattcc atgtggcatc 9480
aggggtacaga accaagcgcg ccagggtaaa atttcaattg gagcctaogc 9540
cgcgaggtgg gcactgtcaa ctgaacggcc aactgcgtgg tatgcgatcg tgcggcgata 9600
tattgtaaag aggtctctca gotcaacaga agtgttcttg tgcgcagggg gtgttgtgtc 9660
ttccacotca gtgcagacca tttgtgcact agagggatgt aaacctotgt tcaacttctt 9720
acaaattcgg tcagtcattg ggcccggtgt atgggcttag tgtggtoact gatttcaaat 9780
totattcaga ctattattgc tgatttttget atttctgtga ttgatgcagc gcttttcttt 9840
otcatgotac ttgcattggc tgttgttact gtgtttcttt tctggctcat tgttgccatc 9900
ggcgcagctg tgggtggcgcg gtgttcacga ggtgcgcgtt acagaactgt ttaaggattt 9960
gagcgtcgac aaactgcgcg cgaaagatgc ottccggagt ctgggatatg ctctgtcgat 10020
tggccagtcg aggtctatcg atatgctgca ggtatgggtg cttgctgcgc accgcaaggga 10080
agttatgcct tocaatatca tgccatggcc cggctctact cctgattgct ttgaccatct 10140
ggagtcttct agctatgotc catttatcaa tgcctatcgg caggcaattt tgagtcaata 10200
cccacaagag ctccagctcg aagccatcaa ctgtaaatg ottgctgtgg ttgcaccggc 10260
attgtatcat aattaccatc tagccaattt gaccggaccg gccacatggg tctgtccctac 10320
agtggggcag ttgcactatt atgcttcttc ctctattttt gcttcactct tgggaagtgtt 10380
ggcagcaata atactactat ttgcattgcat accactagtg aacagagtgt acatctcttt 10440
tacgcggtga atgtcacctt cccgtcgcac ttccagcggc actttgcgcg ggcgcaagat 10500
tttgttagtc acaoggggta tgaatatgcc tgggtcacta tgttagtgca cttgtttgcc 10560
aaactgggtt tgacatttcc gagcttagtt aattgttccc gccctgtgaa tgtctttgct 10620
aatgcttctt gcgtgcaagt ggtttgtagt cataccaact caaatactgg ottgggtcaa 10680
ctttcttttt ctttgttaga tgaagatcta cggctgcata tcaggcctac tcttatttgt 10740
tgggtttgct tgttgttggt gcacttctta cccatgccac gctgcagagg ctctgaattt 10800
tacttaactt agtcatggat tgggcoacgt gcacggtcac gagggtgtga ggaattttat 10860
taatgtcact cattctgcac ttctttatct taatcccacc actcccactg ogcgggctat 10920
aactcattgt ttacttcttg ttctggcagc caaatgggaa caccaaaacg ctactatctg 10980
gctgcagctg cagcgttttg ggtatcatgt ggtggcgat gtcattgtca acttgggaag 11040
ggacaagagg catcttact ttaaaccttt gagagcgccg gctttaocgc ttgggtttgt 11100
ggctatagtt tatgttcttt tacgactggg acgttgggct caacgatgtt atctatgatt 11160
gtattgotat tcttgotttg ggggtgcgca tcacatgctt acttctoata ctacaccgct 11220
cagcgttcoa cagacttcac cttgtgtatg ctgacggatc gcggcggtat tggcaatttg 11280
ctgcgatatg atgagcacac tgetttgtac aattgttccg ccagtaaaac ctggttggtat 11340
tgcacattcc tggacgaaca gattatcaog tctggaaacc attgtgatga cacctacgag 11400
gtcccagttg ctgaggtcct ggaacaggcg catgtgacgt acagtgcgct gtttgatgac 11460
atgccccctt ttatttacta tggccgtgaa ttccggcatag ttgtgttgga tgtgtttatg 11520
ttctatocog ttttagttct gtttttctta toagtaactac octatgctac gcttattctt 11580
gaaatgtgtg tatctattot gtttataato tatggcattt acagcggggc ctacttggcc 11640
atgggoatat ttgcggccac gcttgcata cattcaattg tggtcctcog ocaattactg 11700
tggttatgoc tggcttggcg ataccgctgt acgcttcacg cgtcctttat atcagctgag 11760
gggaaagtgt aocccgtaga ccocggactc ccgttggcog ccgtgggcaa tccgttgtta 11820
gtcccaggta ggccactat cgattatgca gtggcctacg gcagcaaagt caaacttgtg 11880
aggttggggg cagctgaggt atgggagcca tagattcatt ttgtgggtgac gggatttttag 11940
gtgagtatct agattacttt attctgtcog tcccactctt gctgttgctt actaggtatg 12000
tagcatotgg gttagtgtat gttttgactg ccttgttota ttcocttgta ttagcagctt 12060
atatttgggt tgttatagtt ggaagagcct tttctactgc ttatgctttt gtgcttttgg 12120
ctgcttttct gttattagta atgaggatga ttgtgggtat gatgcctcgt cttcgggtcca 12180
ttttcaacca tcgocaaact gtggttagctg attttgtgga cacacctagt ggacctgttc 12240
ccatcccccg ctcaactact caggtagtgg ttccgcggcaa cgggtacacc gcagttggta 12300
acaagcttgt ogatggcgto aagacgataa cgtccgcagg ccgactcttt tcgaaacgga 12360
cggcggcgac agctacaaag ctacaatgac ctactgcgca tgtttgggtca gatgcgggtc 12420
cgcaaacgcg ccgcgcaacc cactcaggct attattgcag agcctggaga ccttaggcac 12480
gattttaaate aacaggagcg ogocaccott tctgcgaacg tacaacgggt cttcatgatt 12540
gggcattggt cactcactgc agatgccgga ggactcacgt acaccgtcag ttgggttccct 12600
acaaacaaa tcoagcgcaa agttgcgcct ccagcagggg ogtaagacgt ggatattcto 12660
ctgtgtggcg tcatgttgaa gtagttatta gccaccacag aacc 12704

```

&lt;210&gt; 11

&lt;211&gt; 33

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

<223> Description of Artificial Sequence:oligonucleotide  
primer

&lt;400&gt; 11

gggtctagag tcaccacaaa atgaatctat ggc

33

&lt;210&gt; 12

&lt;211&gt; 34

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

<223> Description of Artificial Sequence:oligonucleotide  
primer

&lt;400&gt; 12

gggggggatcc cctttgtaga tgaagatcta cggc

34

&lt;210&gt; 13

&lt;211&gt; 31

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

<223> Description of Artificial Sequence:oligonucleotide  
primer

&lt;400&gt; 13

gggggatcct ggtacgttgg gctcaacgat g

31

&lt;210&gt; 14

&lt;211&gt; 43

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

<223> Description of Artificial Sequence:oligonucleotide  
primer

&lt;400&gt; 14

gggggtctag agcaatacaa tcatagataa catogttgag ccc

43

&lt;210&gt; 15

&lt;211&gt; 32

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

<223> Description of Artificial Sequence:oligonucleotide  
primer

&lt;400&gt; 15

gggtctagac cacacaggag aatatccacg tc

32

&lt;210&gt; 16

&lt;211&gt; 33

&lt;212&gt; DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:oligonucleotide primer

<400> 16

gggggggatcc cagctgaggt atgggagoca tag

33

<210> 17

<211> 32

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:oligonucleotide primer

<400> 17

gggggatccc gcagttggta acaagcttgt cg

32

<210> 18

<211> 34

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:oligonucleotide primer

<400> 18

ggggggaatt ccattgcgcag taggtcattg tage

34

<210> 19

<211> 33

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:oligonucleotide primer

<400> 19

gggtctagag toaccacaaa atgaatctat ggc

33

<210> 20

<211> 33

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:oligonucleotide primer

<400> 20

gggggggtcg actggctccc atacctcage tgc

33

<210> 21

<211> 31

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:oligonucleotide  
primer

<400> 21

gggggatacct ggtacgttgg gctcaacgat g

31

<210> 22

<211> 36

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:oligonucleotide  
primer

<400> 22

ggggggagat ctatggcgtc aagacgatca cgtcgcg

36

<210> 23

<211> 31

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:oligonucleotide  
primer

<400> 23

gggggatoca tttctgtgat tgatgcagcg c

31

<210> 24

<211> 33

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:oligonucleotide  
primer

<400> 24

gggggggtcg accggccctg ctggaggcgc aac

33

<210> 25

<211> 36

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:oligonucleotide  
primer

<400> 25

gggtctagag gcatattcat aaccggtgtg cactac

36



<210> 26  
<211> 36  
<212> DNA  
<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:oligonucleotide  
primer

<400> 26  
ggggggagat ctatgcagcg cttttcttcto tcatgc

36

<210> 27  
<211> 34  
<212> DNA  
<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:oligonucleotide  
primer

<400> 27  
gggggatcct ttgaccggac cggccacatg ggto

34

<210> 28  
<211> 35  
<212> DNA  
<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:oligonucleotide  
primer

<400> 28  
ggggggggtcg accaaaatct tgcgcgcgcg caaag

35

<210> 29  
<211> 36  
<212> DNA  
<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:oligonucleotide  
primer

<400> 29  
gggtctagag taagtaaaat tacgagcctc tgcago

36

<210> 30  
<211> 38  
<212> DNA  
<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:oligonucleotide  
primer

<400> 30

gggggggagat ctatgggagc catagattca ttttgtgg

38

&lt;210&gt; 31

&lt;211&gt; 33

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

<223> Description of Artificial Sequence:oligonucleotide  
primer

&lt;400&gt; 31

gggggatccc ctttgtagat gaagatctac ggc

33

&lt;210&gt; 32

&lt;211&gt; 35

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

<223> Description of Artificial Sequence:oligonucleotide  
primer

&lt;400&gt; 32

ggggggggtcg acttgtagct tgtaggctgt cgccg

35

&lt;210&gt; 33

&lt;211&gt; 41

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

<223> Description of Artificial Sequence:oligonucleotide  
primer

&lt;400&gt; 33

gggtctagag caatacaatc atagataaca tcgttgagcg a

41

&lt;210&gt; 34

&lt;211&gt; 35

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

<223> Description of Artificial Sequence:oligonucleotide  
primer

&lt;400&gt; 34

ggggggggaat tcatgggaac cttctccgct actgg

35

&lt;210&gt; 35

&lt;211&gt; 32

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

<223> Description of Artificial Sequence:oligonucleotide  
primer

<400> 35  
gggggatccc agctgaggta tgggagccat ag 32

<210> 36  
<211> 36  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence:oligonucleotide  
primer

<400> 36  
gggggggtcta gatcacacgg gcccaatgac tgaac 36

<210> 37  
<211> 32  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence:oligonucleotide  
primer

<400> 37  
ggggaattcc atgggcagta ggtcattgta gc 32

<210> 38  
<211> 38  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence:oligonucleotide  
primer

<400> 38  
ccccttaagt gccatatacg gctcaccacc atatacac 38

<210> 39  
<211> 32  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence:oligonucleotide  
primer

<400> 39  
gggggggatcc tggtaggttg ggctcaacga tg 32

<210> 40  
<211> 37  
<212> DNA  
<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:oligonucleotide  
primer

<400> 40

cccttcaggga ggttcctggg tggctaataa ctacttc

37

<210> 41

<211> 35

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:oligonucleotide  
primer

<400> 41

gggggtctag agtcaccaca aaatgaatct atggc

35

<210> 42

<211> 22

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:oligonucleotide  
primer

<400> 42

gggggggatcc cagtactttg gc

22

<210> 43

<211> 33

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:oligonucleotide  
primer

<400> 43

gggggggatcc cgcagttggt aacaagcttg tcg

33

<210> 44

<211> 29

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:oligonucleotide  
primer

<400> 44

gggggggatcc cagcgacacc cgaggcacg

29

<210> 45

<211> 34

<212> DNA

<213> Artificial Sequence

&lt;220&gt;

<223> Description of Artificial Sequence:oligonucleotide  
primer

&lt;400&gt; 45

gggggtctag accacacagg agaatatcca cgtc

34

&lt;210&gt; 46

&lt;211&gt; 36

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

<223> Description of Artificial Sequence:oligonucleotide  
primer

&lt;400&gt; 46

ggggggcatg ctgtaggtgg gccatggatc aagtc

36

&lt;210&gt; 47

&lt;211&gt; 33

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

<223> Description of Artificial Sequence:oligonucleotide  
primer

&lt;400&gt; 47

gggggtcgacg tcaccacaaa atgaatctat ggc

33

&lt;210&gt; 48

&lt;211&gt; 33

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

<223> Description of Artificial Sequence:oligonucleotide  
primer

&lt;400&gt; 48

ggggggcatg ccacctgggc caagaaattg acc

33

&lt;210&gt; 49

&lt;211&gt; 31

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

<223> Description of Artificial Sequence:oligonucleotide  
primer

&lt;400&gt; 49

ggggaattcc acacaggaga atatccacgt c

31

&lt;210&gt; 50

&lt;211&gt; 24

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:oligonucleotide  
primer

<400> 50

gctggccgcg taatgctggt tggg

24

<210> 51

<211> 22

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:oligonucleotide  
primer

<400> 51

caactatgoc gaattoacgg cc

22

<210> 52

<211> 26

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:oligonucleotide  
primer

<400> 52

ccgtgctgga tgtgaggccg ttaccg

26

<210> 53

<211> 22

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:oligonucleotide  
primer

<400> 53

ggccgtgaat toggcatagt tg

22

<210> 54

<211> 37

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:oligonucleotide  
primer

<400> 54

gggggggaccc atgttatctc tgattgtatt gctattc

37

<210> 55  
<211> 32  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence:oligonucleotide  
primer

<400> 55  
gggggggatcc atttctgtga ttgatgcagc gc 32

<210> 56  
<211> 35  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence:oligonucleotide  
primer

<400> 56  
gggggggtcta gacaacacaa ctatgccgaa ttcac 35

<210> 57  
<211> 38  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence:oligonucleotide  
primer

<400> 57  
ggggggtctag aggcattatc ataaccogtg tgcactac 38

<210> 58  
<211> 23  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence:oligonucleotide  
primer

<400> 58  
atggtatggc tagcatgact ggt 23

<210> 59  
<211> 35  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence:oligonucleotide  
primer

<400> 59  
gggggggatcc ttggaccgga ccggccacat gggtc 35

<210> 60  
<211> 23  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence:oligonucleotide  
primer

<400> 60  
caaacaacag atggctggca act

23

<210> 61  
<211> 38  
<212> DNA  
<213> Artificial Sequence

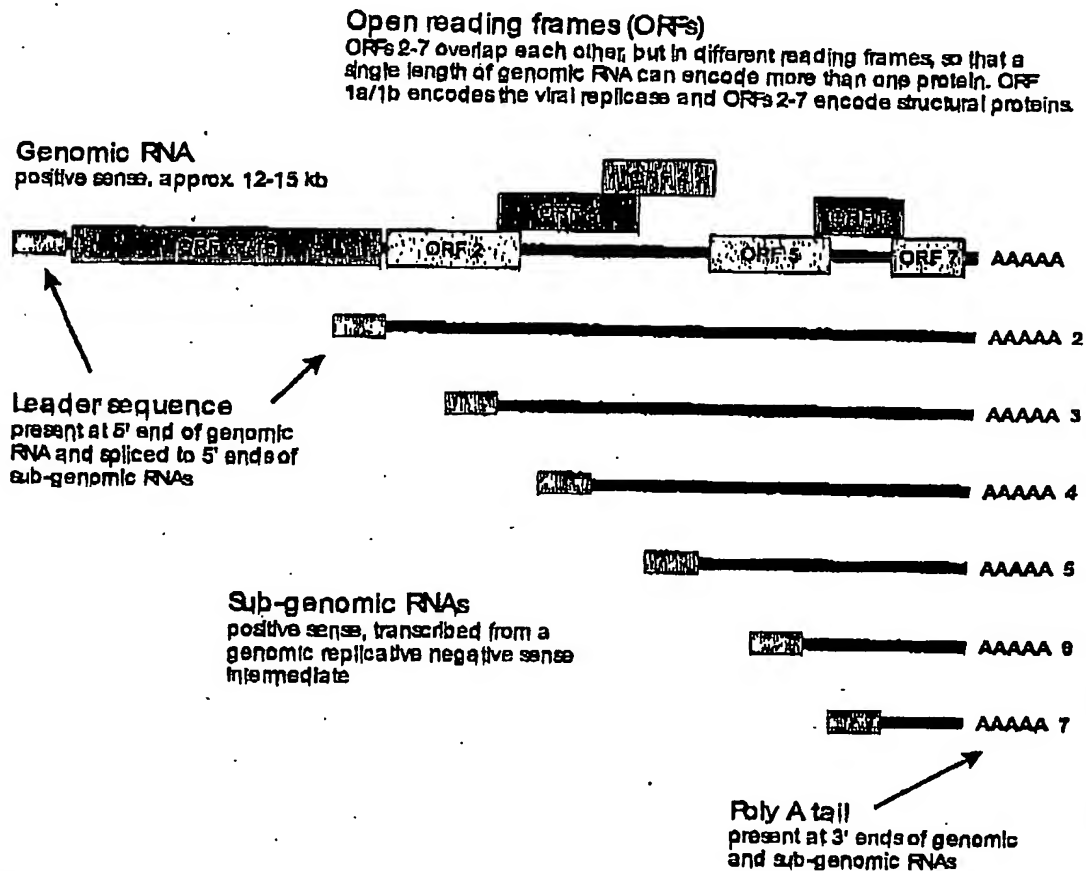
<220>  
<223> Description of Artificial Sequence:oligonucleotide  
primer

<400> 61  
gggggtctag agtaagtaaa attacgagcc totgcagc

38



# Genome organization and transcription of arteriviruses



**Fig. 1**

Schematic diagram of the genomic organization and transcriptional strategy of the family *Arteriviridae*.

## Construction of a vector expressing the neutralization determinant of the viral large glycoprotein

The N-terminal hydrophilic ectodomain (amino acids 1-121) of the G(L) envelope glycoprotein contains the neutralization domains of EAV (Balasuriya et al., 1997, Virology 232, 114-128). The corresponding coding region of the viral ORF 5 (nucleotides 1-363) was inserted into the mammalian expression vector pcDNA3.1/His.

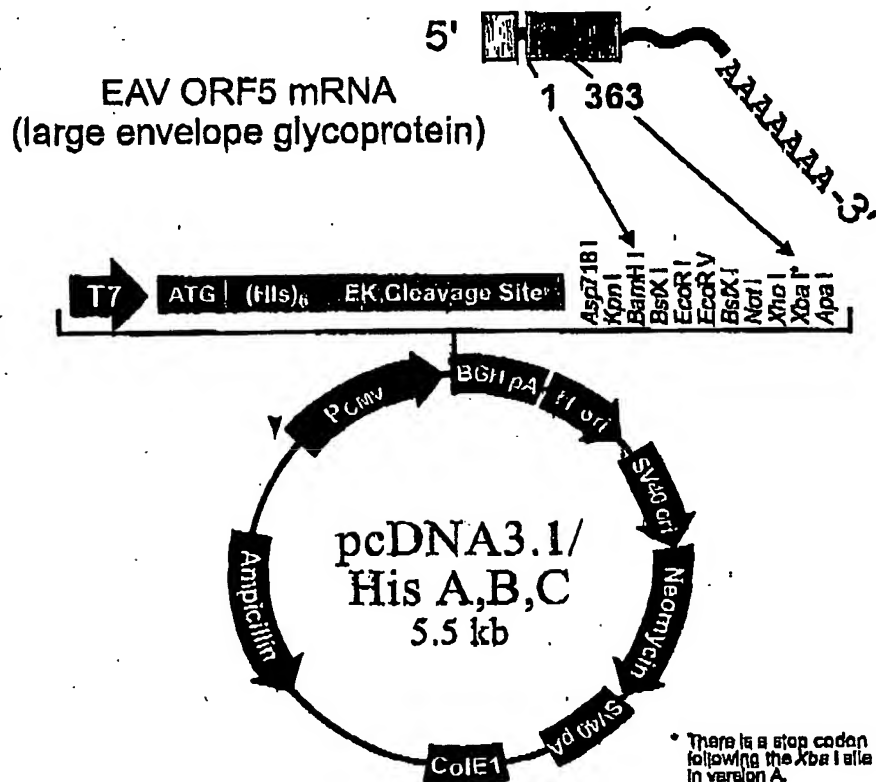


Fig. 2:

Schematic diagram of the strategy used for molecular cloning of neutralizing domain of equine arteritis virus (EAV). A part of the cDNA of viral ORF5 expressing the N-terminal hydrophilic ectodomain of the EAV envelope large glycoprotein was inserted into the corresponding sites of mammalian expression vector pcDNA3.1.

**A**

Diagram presenting the data of DNA immunization of Balb/c mice  
with recombinant plasmid pCR3.1-EAV-OS-BX-C14  
expressing ORF 5 of equine arteritis virus

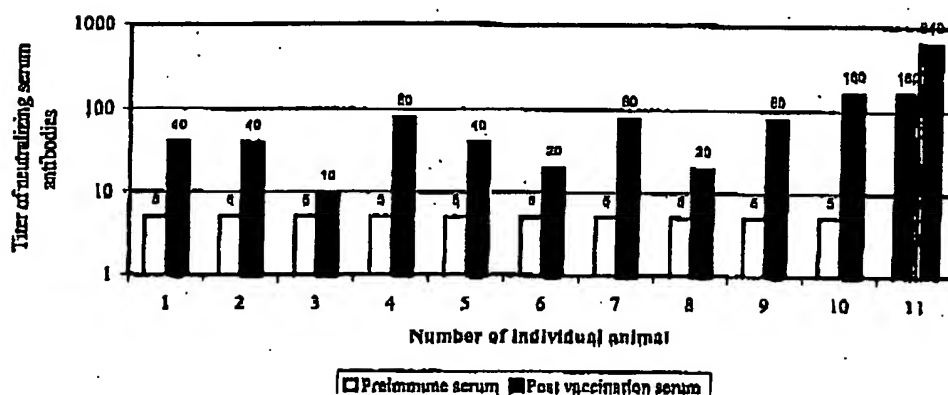
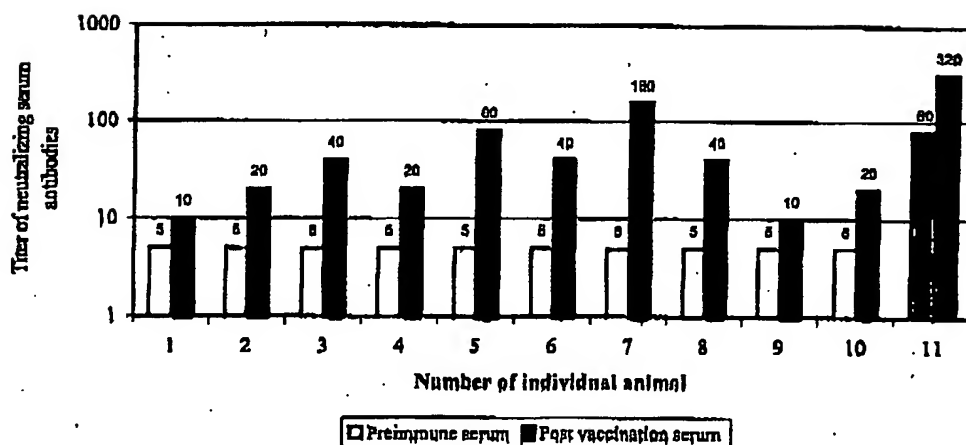
**B**

Diagram presenting the data of DNA immunization of Balb/c mice  
with recombinant plasmid pCR3.1-EAV-OS-BX-C14  
expressing the ORF 5 of equine arteritis virus

**Fig. 3:**

The results of neutralization tests obtained by the analysis of the sera of the individual Balb/c mice that were inoculated in two independent experiments (A and B) with the DNA of recombinant plasmid pCR3.1-EAV-OS-BX-C14 harboring and expressing ORF 5 of equine arteritis virus (EAV). The individual animals are indicated with number 1 to 10. The white and black columns represent the data of pre and post DNA vaccinated animals, respectively. The column 11 (red color) served as internal positive control and indicates the average of maximum and minimum neutralizing titer obtained from the serum of a New Zealand white rabbit that was immunized with inactivated EAV.

Diagram presenting the data of DNA immunization of Balb/c mice with recombinant plasmids pCR3.1-EAV-O5-BX-C14 and pCR3.1-EAV-O7-BX-C3 expressing ORF 5 and 7 of equine arteritis virus

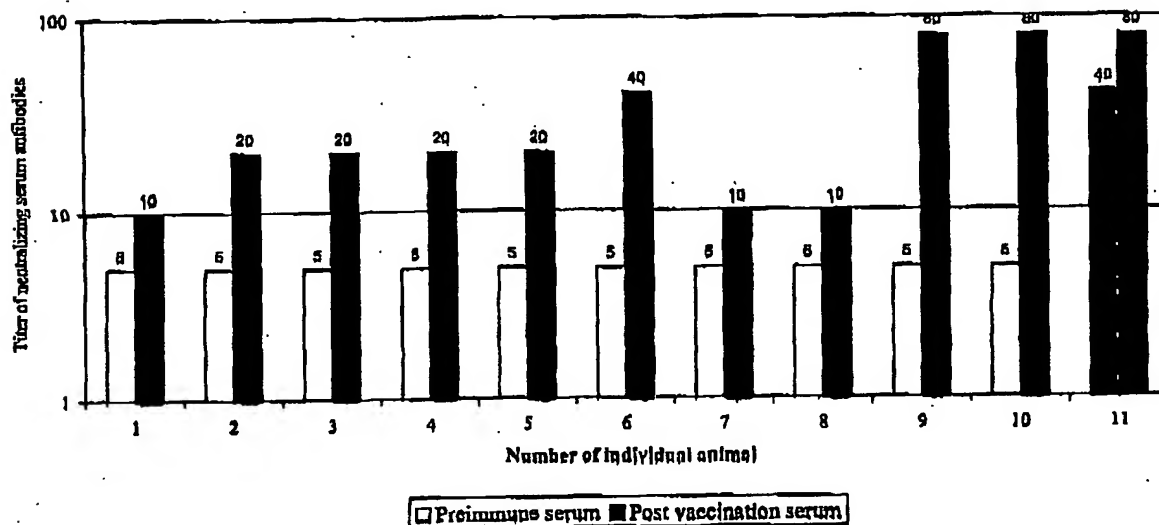


Fig. 4:

The results of neutralization test obtained by the analysis of the sera of the individual Balb/c mice that were inoculated with the DNA of recombinant plasmids pCR3.1-EAV-O5-BX-C14 and pCR3.1-EAV-O7-BX-C3 harboring and expressing ORFs 5 and 7 of equine arteritis virus (EAV). The individual animals are indicated with number 1 to 10. The white and black columns represent the data of pre and post DNA vaccinated animals, respectively. The column 11 (red color) served as internal positive control and indicates the average of maximum and minimum neutralizing titer obtained from the serum of a New Zealand white rabbit that was immunized with inactivated EAV.

Diagram presenting the data of DNA immunization of Balb/c mice with recombinant plasmids pDP-EAV-O7-BsS-C2 and pDP-EAV-O5-BsS-C1 expressing ORF 7 and 5 of Equine arteritis virus, as well as pWS2ms expressing IL-2 gene

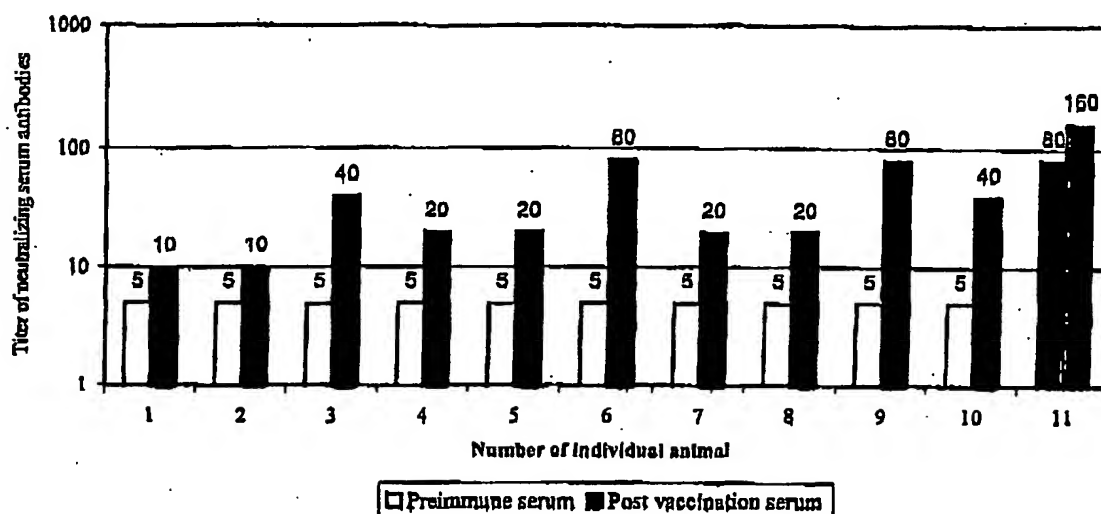
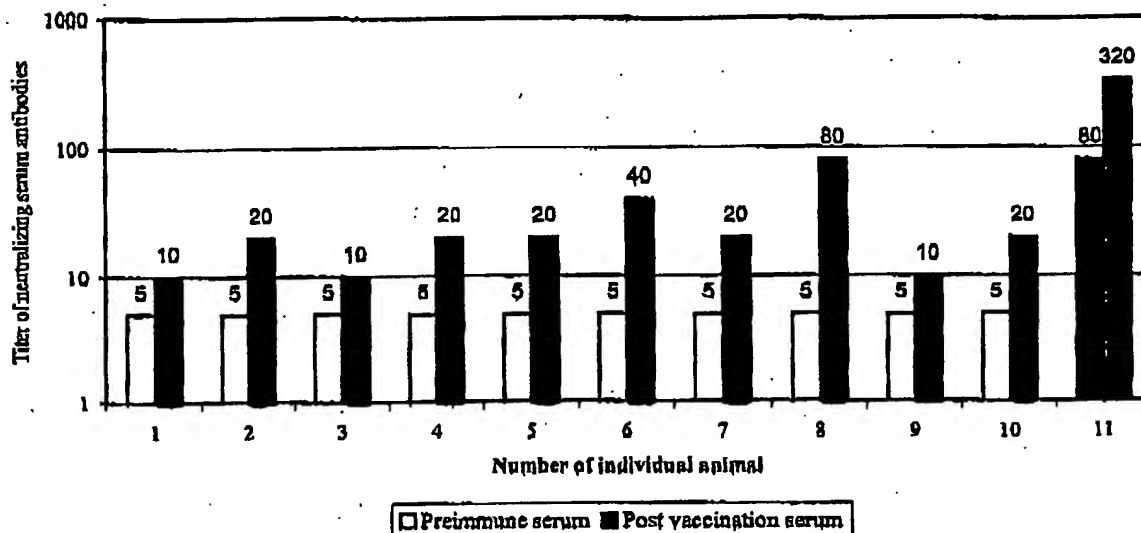


Fig. 5:

The results of neutralization test obtained by the analysis of the sera of the individual Balb/c mice that were inoculated with the DNA of recombinant plasmids pDP-EAV-O5-BsS-C2 and pDP-EAV-O7-BsS-C1 harboring and expressing ORFs 5 and 7 of equine arteritis virus (EAV). The recombinant plasmid pWS2ms expressing mouse IL2 gene was administered as immune modulating factor. The individual animals are indicated with number 1 to 10. The white and black columns represent the data of pre and post DNA vaccinated animals, respectively. The column 11 (red color) served as internal positive control and indicates the average of maximum and minimum neutralizing titer obtained from the serum of a New Zealand white rabbit that was immunized with inactivated EAV.

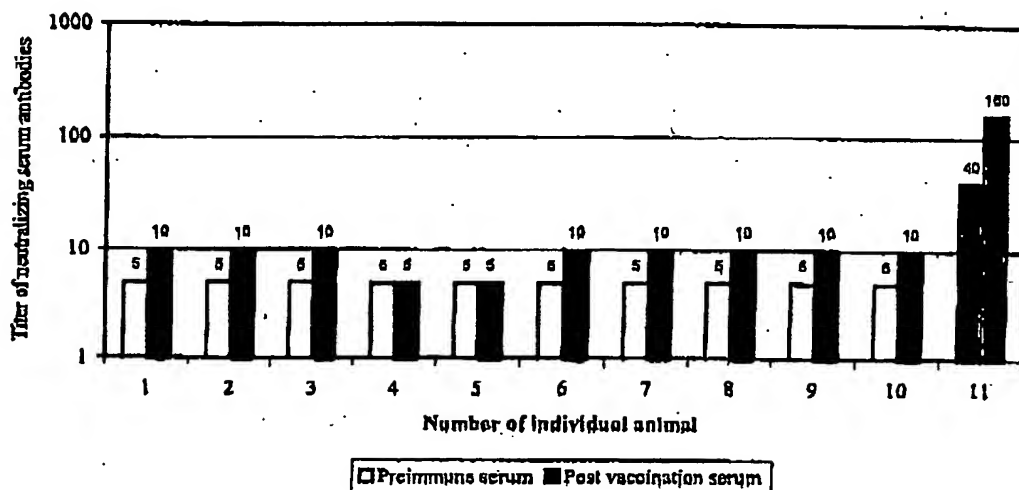
Diagram presenting the data of DNA immunization of Balb/c mice with recombinant plasmids pCR3.1-EAV-O5-BX-C14 and pCR3.1-EAV-O6-BE-C4 expressing ORF 5 and 6 of equine arteritis virus



**Fig. 6:**

The results of neutralization test obtained by the analysis of the sera of the individual Balb/c mice that were inoculated with the DNA of recombinant plasmids pCR3.1-EAV-O5-BX-C14 and pCR3.1-EAV-O6-BE-C4 harboring and expressing ORFs 5 and 6 of equine arteritis virus (EAV). The individual animals are indicated with number 1 to 10. The white and black columns represent the data of pre and post DNA vaccinated animals, respectively. The column 11 (red color) served as internal positive control and indicates the average of maximum and minimum neutralizing titer obtained from the serum of a New Zealand white rabbit that was immunized with inactivated EAV.

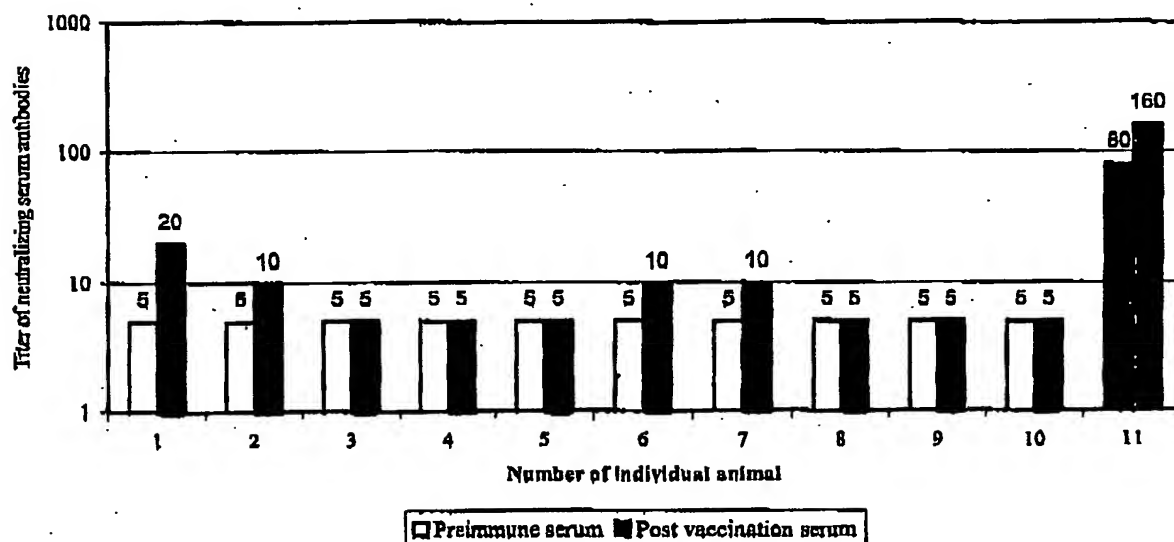
Diagram presenting the data of DNA immunization of Balb/c mice  
with recombinant plasmids pCR3.1-EAV-O3-BX-C1  
expressing ORF 3 of equine arteritis virus



**Fig. 7:**

The results of neutralization test obtained by the analysis of the sera of the individual Balb/c mice that were inoculated with the DNA of recombinant plasmid pCR3.1-EAV-O4-BX-C3 harboring and expressing ORF 4 of equine arteritis virus (EAV). The individual animals are indicated with number 1 to 10. The white and black columns represent the data of pre and post DNA vaccinated animals, respectively. The column 11 (red color) served as internal positive control and indicates the average of maximum and minimum neutralizing titer obtained from the serum of a New Zealand white rabbit that was immunized with inactivated EAV.

**Diagram presenting the data of DNA immunization of Balb/c mice  
with recombinant plasmids pCR3.1-EAV-O4-BX-C3  
expressing ORF 4 of equine arteritis virus**

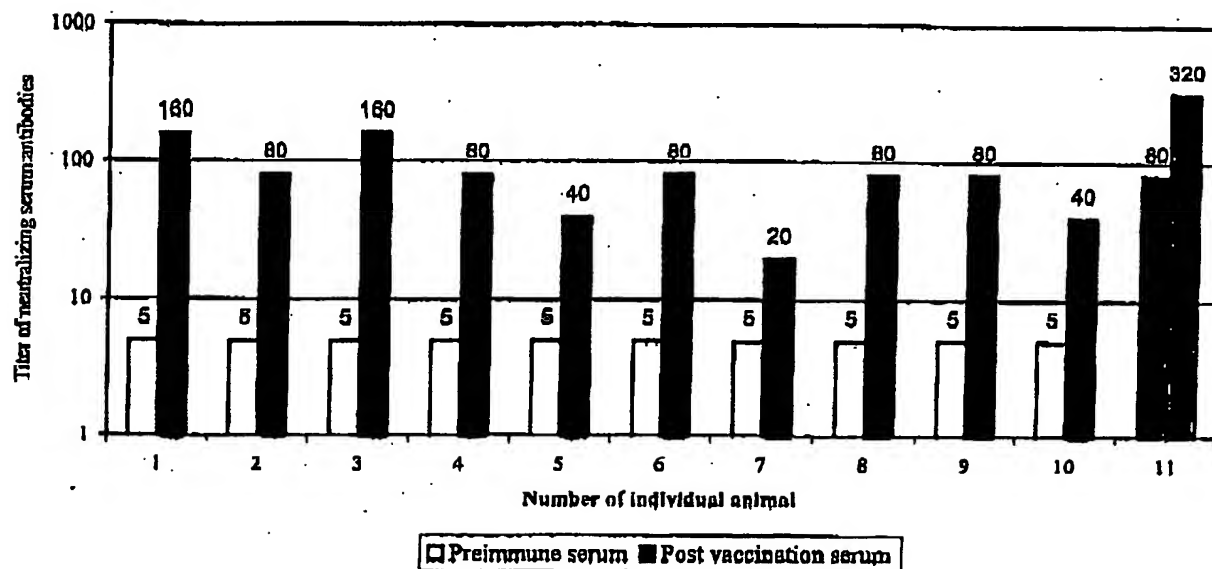


**Fig. 8:**

The results of neutralization test obtained by the analysis of the sera of the individual Balb/c mice that were inoculated with the DNA of recombinant plasmid pCR3.1-EAV-O4-BE-C3 harboring and expressing ORF 4 of equine arteritis virus (EAV). The individual animals are indicated with number 1 to 10. The white and black columns represent the data of pre and post DNA vaccinated animals, respectively. The column 11 (red color) served as internal positive control and indicates the average of maximum and minimum neutralizing titer obtained from the serum of a New Zealand white rabbit that was immunized with inactivated EAV.



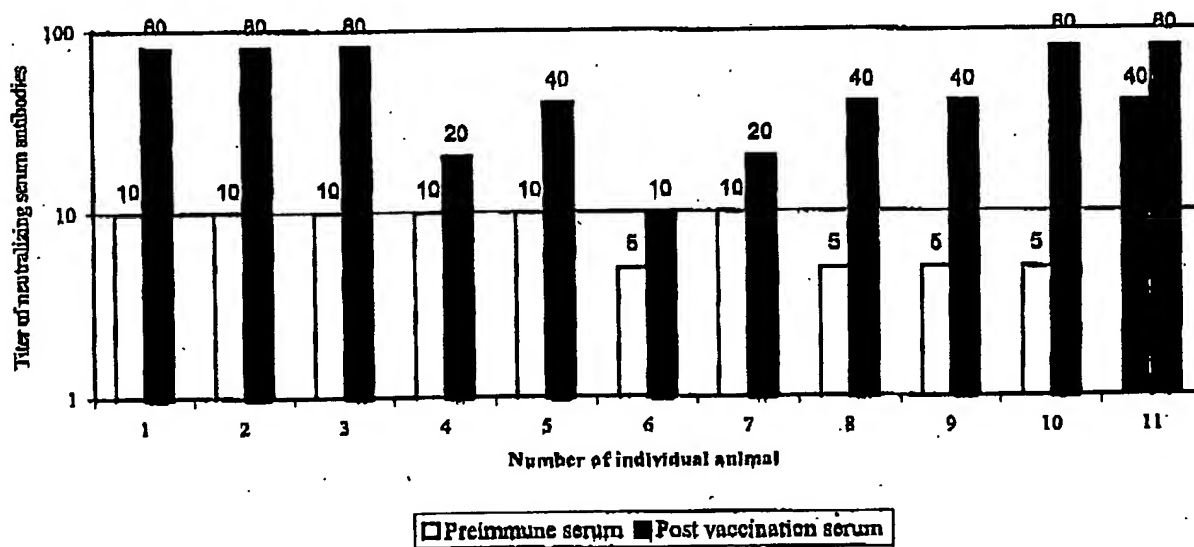
**Diagram presenting the data of DNA immunization of Balb/c mice with recombinant plasmid pC3.1-EAV-O5-del-121 expressing the amino terminus (aa 1-121) of ORF 5 of equine arteritis virus**



**Fig. 9:**

The results of neutralization tests obtained by the analysis of the sera of the individual Balb/c mice that were inoculated with the DNA of recombinant plasmid pCR31-EAV-O5-del-121 harboring and expressing the N-terminal hydrophilic ectodomain of G<sub>L</sub> envelope glycoprotein (amino acid residue 1-121 of ORF 5) of equine arteritis virus (EAV). The individual animals are indicated with number 1 to 10. The white and black columns represent the data of pre and post DNA vaccinated animals, respectively. The column 11 (red color) served as internal positive control and indicates the average of maximum and minimum neutralizing titer obtained from the serum of a New Zealand white rabbit that was immunized with inactivated EAV.

Diagram presenting the data of DNA immunization of Balb/c mice with recombinant plasmids pCR3.1-EAV-O2-BX-C5, pCR3.1-EAV-O5-BX-C14, and pCR3.1-EAV-O6-BE-C4 expressing ORFs 2, 5, and 6 of EAV, as well as pWS-2ms-C1 expressing IL-2



**Fig. 10:**

The results of neutralization test obtained by the analysis of the sera of the individual Balb/c mice that were inoculated with the DNA of recombinant plasmids pCR3.1-EAV-O2-BX-C5, pCR3.1-EAV-O5-BX-C14, and pCR3.1-EAV-O6-BE-C4 harboring and expressing ORFs 2 (small glycoprotein), 5 (large envelope glycoprotein), and 6 (membrane protein), of equine arteritis virus (EAV). The recombinant plasmid pWS2ms (expressing mouse IL2 gene) was administered as immune modulating factor. The individual animals are indicated with number 1 to 10. The white and black columns represent the data of pre and post DNA vaccinated animals, respectively. The column 11 (red color) served as internal positive control and indicates the average of maximum and minimum neutralizing titer obtained from the serum of a New Zealand white rabbit that was immunized with inactivated EAV.

Diagram presenting the data of DNA Immunization of Balb/c mice  
with recombinant plasmids pCR3.1-EAV-O2-BX-C5 and pCR3.1-EAV-O4-BX-C3  
expressing ORF 2 and 4 of equine arteritis virus

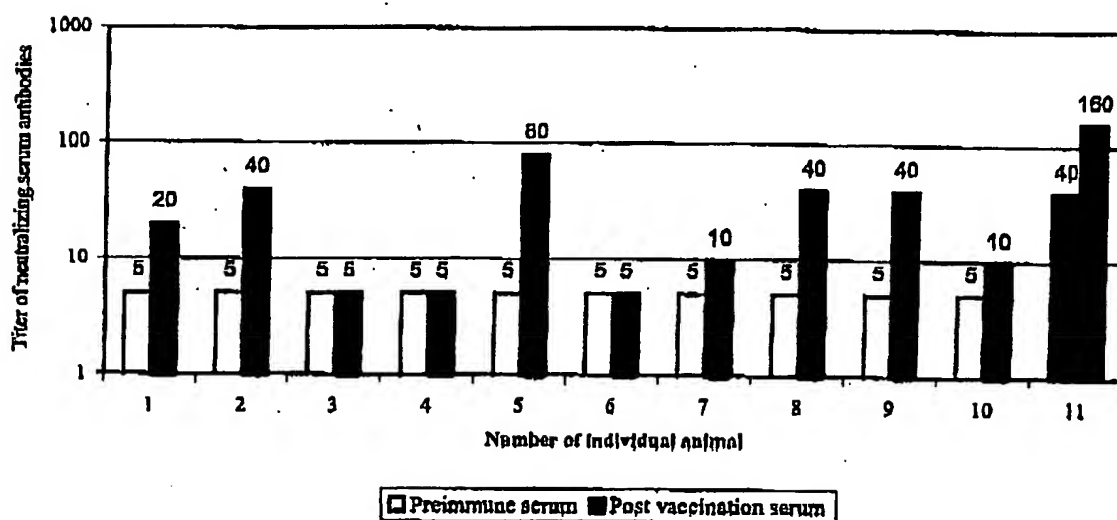


Fig. 11:

The results of neutralization test obtained by the analysis of the sera of the individual Balb/c mice that were inoculated with the DNA of recombinant plasmids pCR3.1-EAV-O2-BX-C5 and pCR3.1-EAV-O4-BX-C3 harboring and expressing ORFs 2 and 4 of equine arteritis virus (EAV). The individual animals are indicated with number 1 to 10. The white and black columns represent the data of pre and post DNA vaccinated animals, respectively. The column 11 (red color) served as internal positive control and indicates the average of maximum and minimum neutralizing titer obtained from the serum of a New Zealand white rabbit that was immunized with inactivated EAV.

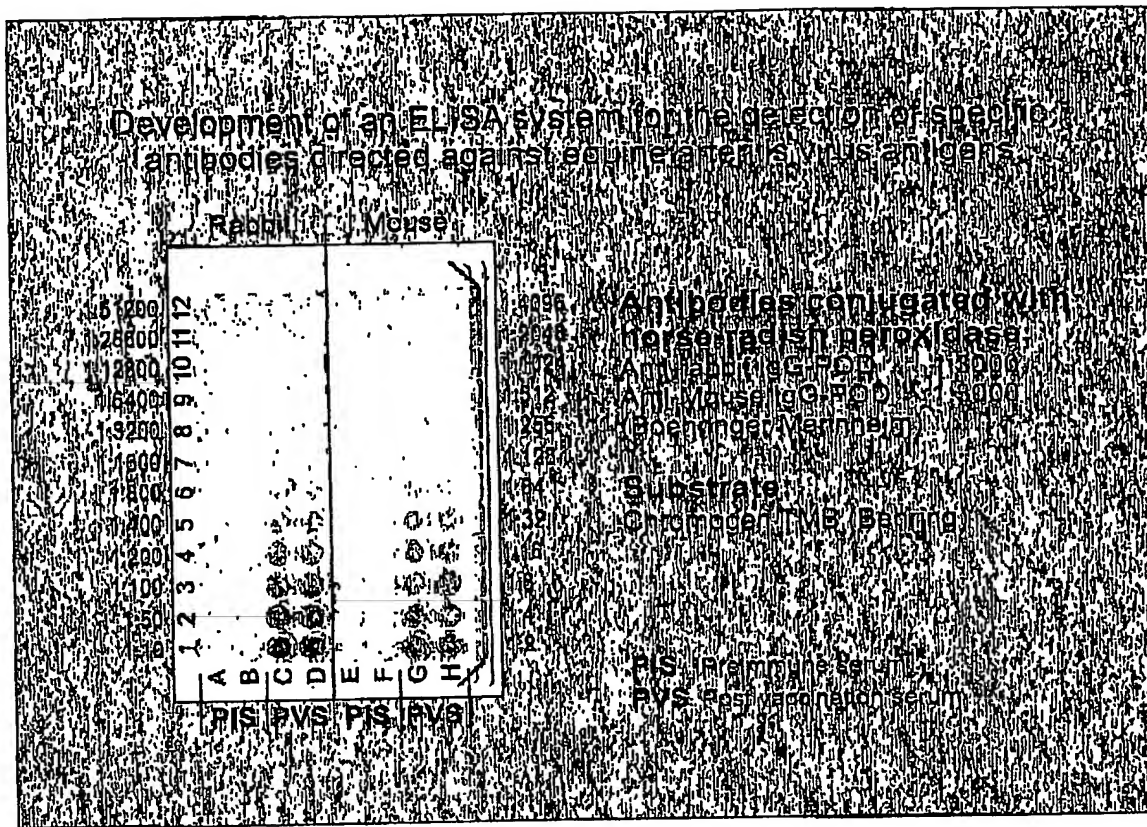
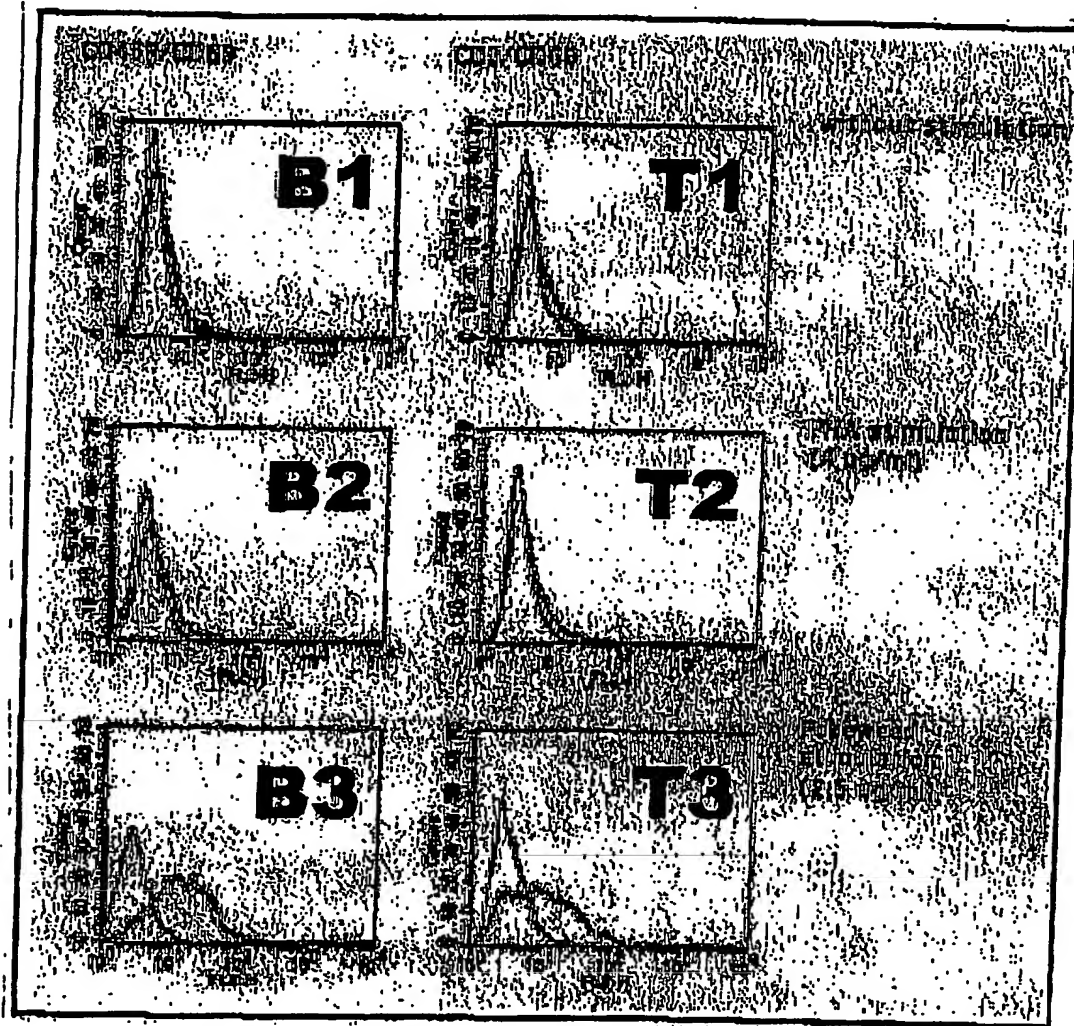


Fig. 12:

An example of the results obtained by enzyme linked immunosorbent assay (ELISA) for the detection of EAV specific antibodies. Polysorp F8 microtiter plates were coated with EAV Protein (EAV+ Host (RK13)) at a concentration of  $2 \mu\text{g} \times \text{ml}^{-1}$  in PBS (+ 0.05%  $\text{N}_3\text{Na}$ ) over night at room temperature (for detail see session material and methods). The assay was stopped after 30 min by the addition of 50  $\mu\text{l}$ /well stopping solution POD and read according to standard procedures at 450 nm on an automatic ELISA reader (MR5000, DYNATECH, Denckendorf, Deutschland). Photograph was taken prior to the analysis of the assay by 450 nm by ELISA reader.

## Establishing of a lymphoproliferation assay for detection of cellular immune response



**Fig. 13:**

Representative FACS profiles of expression of various adhesion molecules on spleen cells from a female BALB/c mouse. B- and T-cells were activated for three days with 8 g/ml PHA (Lectin from *Phaseolus vulgaris* Sigma, Cat. No. L-8754) (Fig. T2 and B2) or 2.5 g/ml Pokeweed (Lectin from *Phytolacca americana*, Sigma, Cat. No. L-9379) (Fig. T3 and B3) and only FACS buffer (PBS, 2% FCS, 0.01% NaN<sub>3</sub>) as negative control (Fig. T1 and B1) respectively. B-cells were stained for single fluorescence with anti-mouse CD45R/ B220 (diluted 1:100 in FACS buffer, RA3-8B2, PharMingen, Cat. No. 01124A, FITC) (Fig. B1, B2 and B3 green lane) and for double fluorescence with anti-mouse CD69 (diluted 1:100 in FACS buffer, H1.2F3, PharMingen, Cat. No. 01505B, PE) and anti-mouse CD45R/ B220 (Fig. B1, B2 and B3, red line, showing the activated B-cells). T-cells were stained for single fluorescence anti-mouse CD3 (diluted 1:100 in FACS buffer, 145-2C11, PharMingen, Cat. No. 01088A, Q-Chrome) (Fig. T1, T2 and T3 green lane) and for double fluorescence with anti-mouse CD69 (diluted 1:100 in FACS buffer, H1.2F3, PharMingen, Cat. No. 01505B, PE) and anti-mouse CD3. Cells were sorted and measured in FACScan (Becton Dickinson) (Fig. T1, T2 and T3, red line, showing the activated T-cells).

Empfangszeit 30. Jan. 10:10

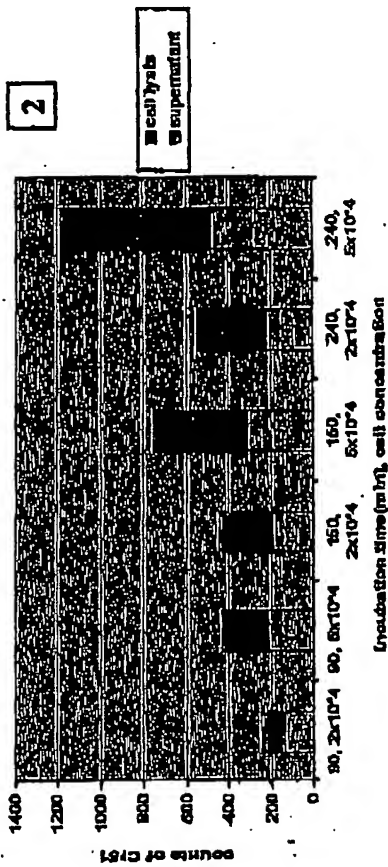
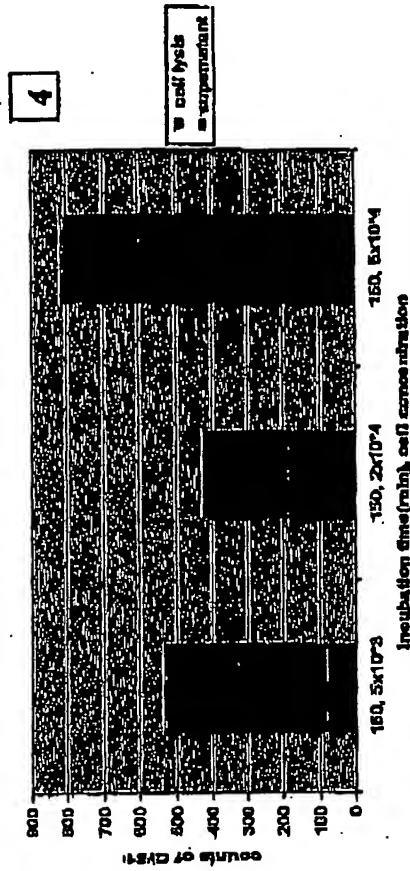
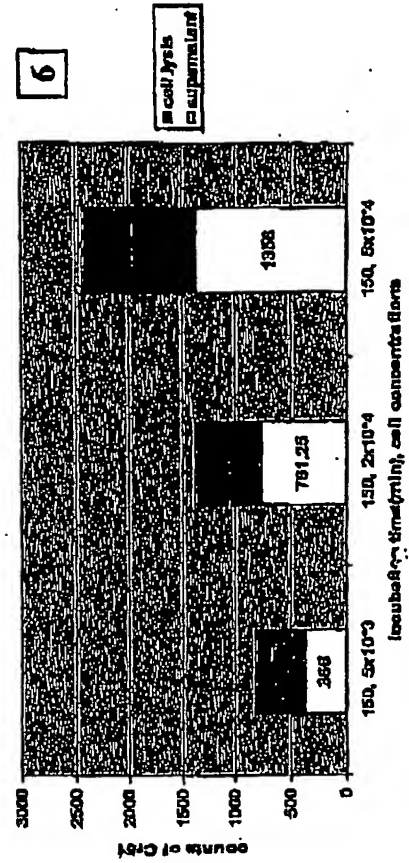
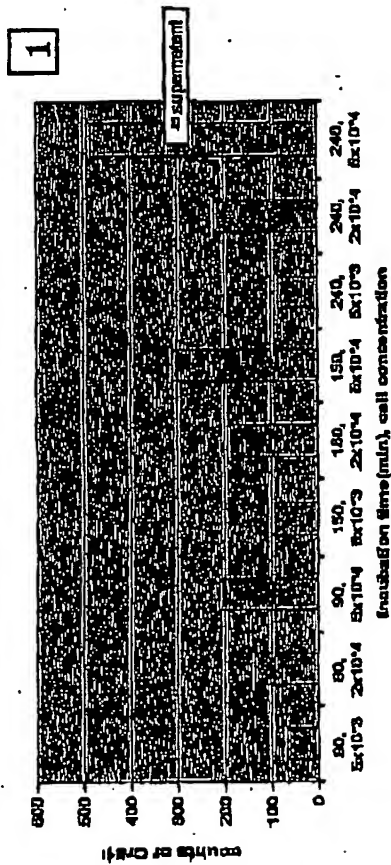
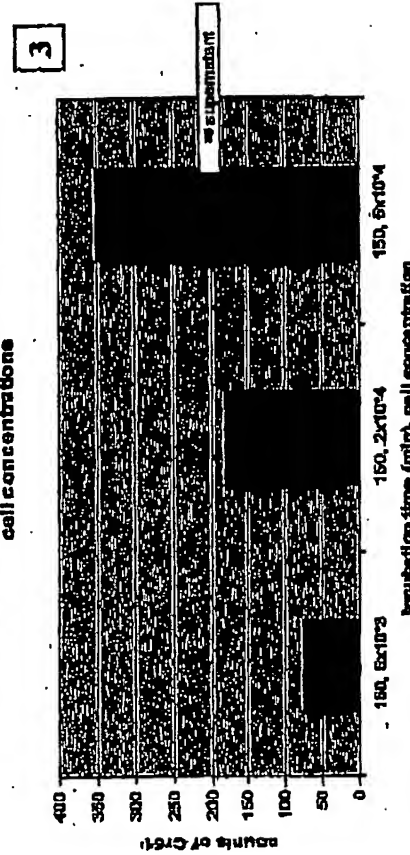
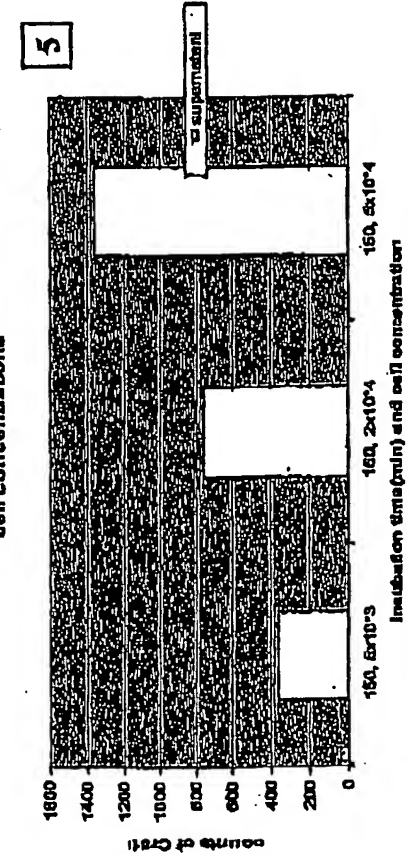
Titration experiment with 100  $\mu\text{Ci}$  Cr51 /  $10^7$  cellsTitration experiment with 200  $\mu\text{Ci}$  Cr51 /  $10^7$  cellsTitration experiment with 400  $\mu\text{Ci}$  Cr51 /  $10^7$  cellsTitration experiment with 100  $\mu\text{Ci}$  Cr51 /  $10^7$  cells and different cell concentrations and incubation timeTitration experiment with 200  $\mu\text{Ci}$  Cr51 /  $10^7$  cells and different cell concentrationsTitration experiment with 400  $\mu\text{Ci}$  Cr51 /  $10^7$  cells and different cell concentrations

Fig. 14 a)



Subtraction (cell lysis - supernatant)

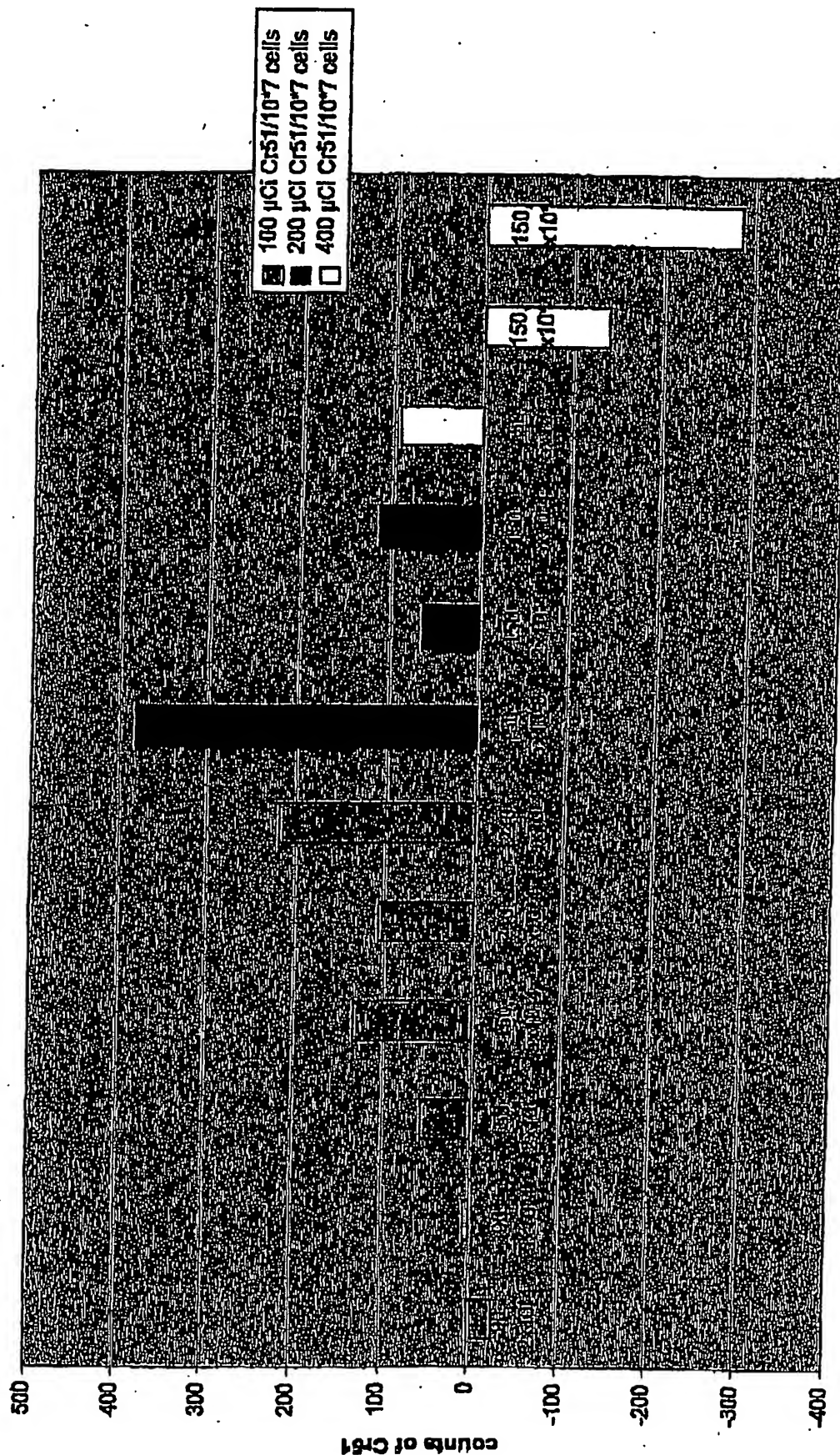
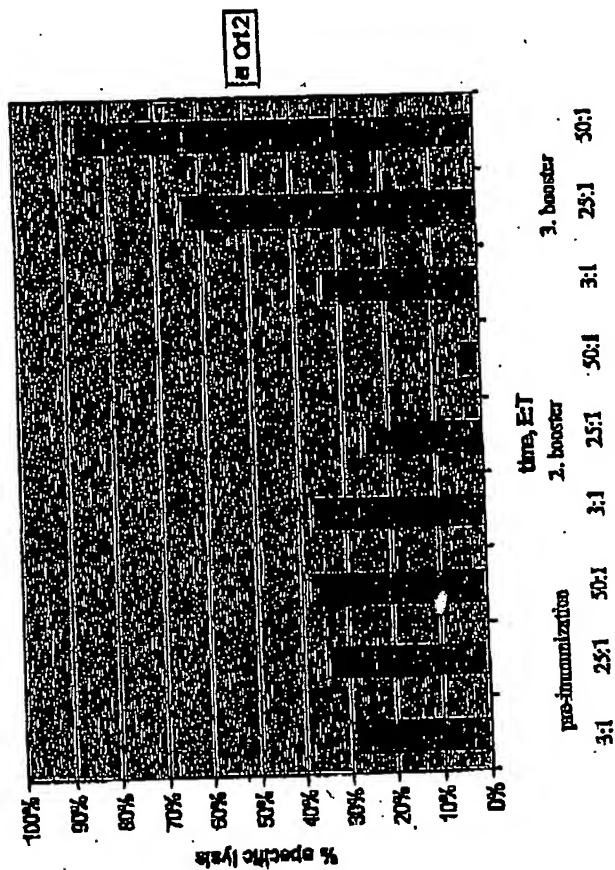
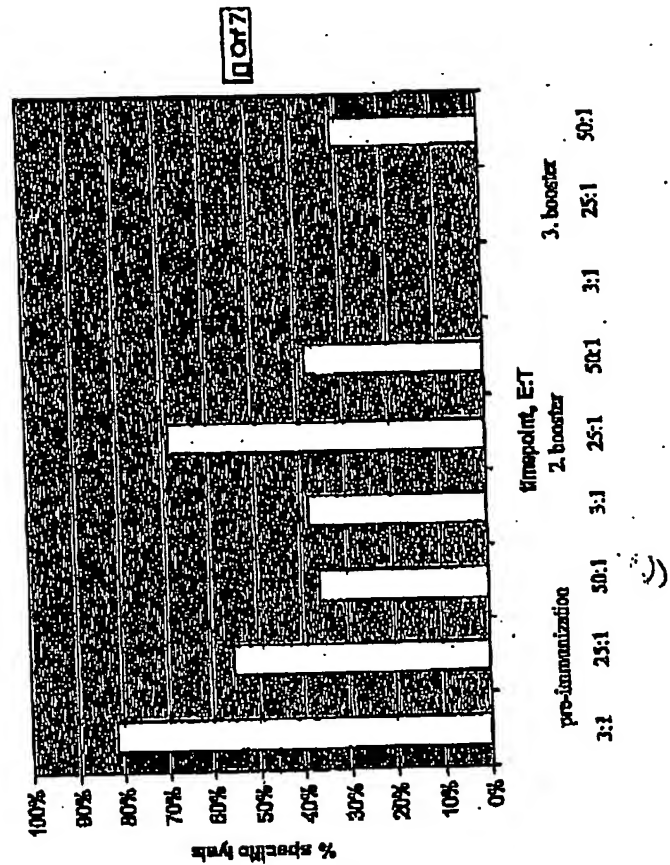


Fig. 14 b)

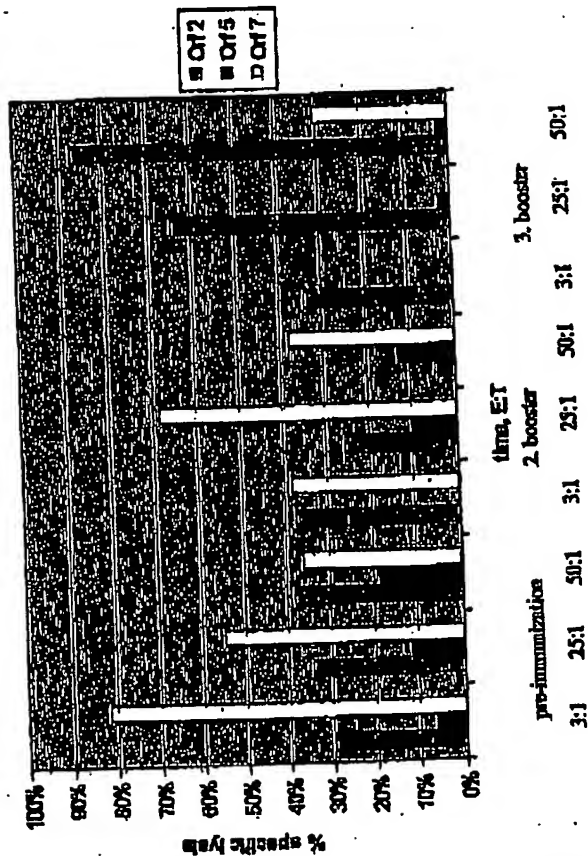
Daggy, specific lysis Orf 2



Daggy, specific lysis Orf 7



Daggy, specific lysis



Daggy, specific lysis Orf 5

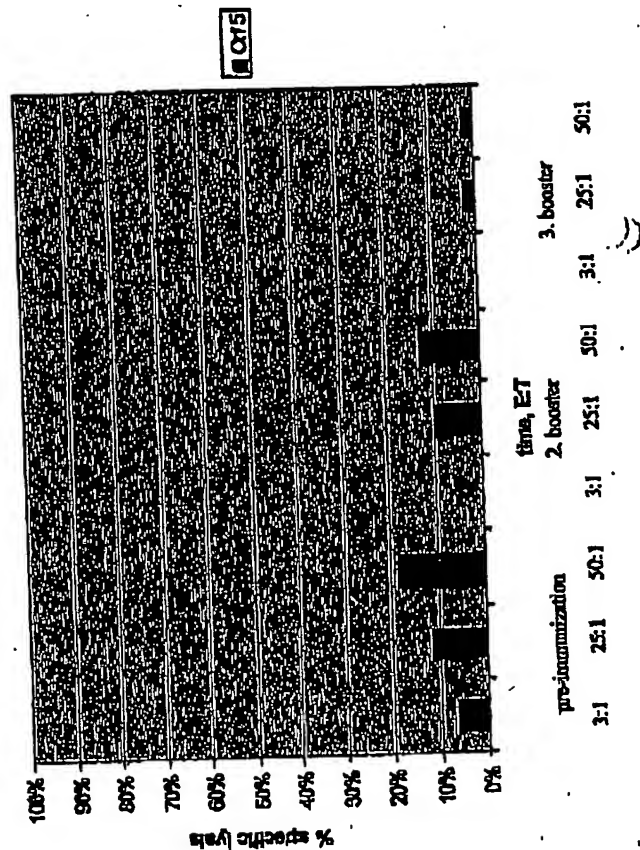
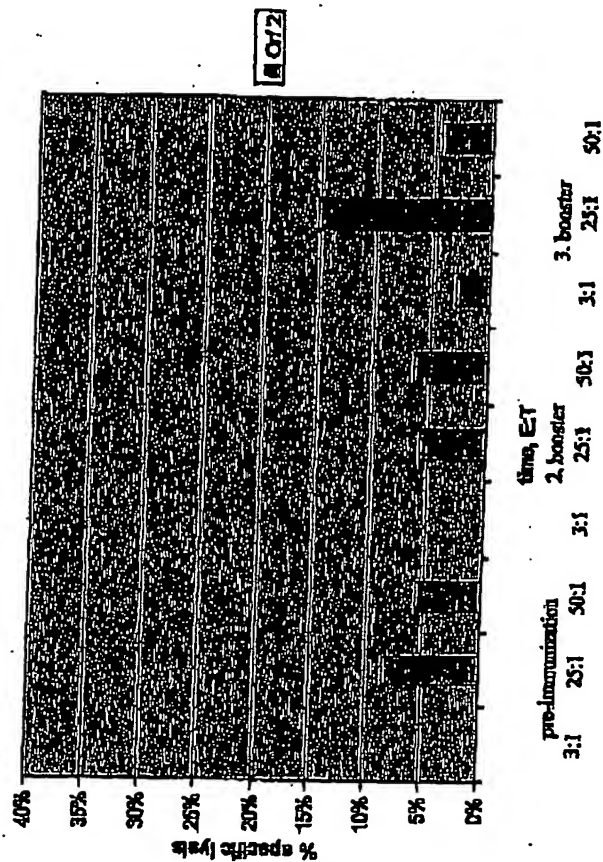


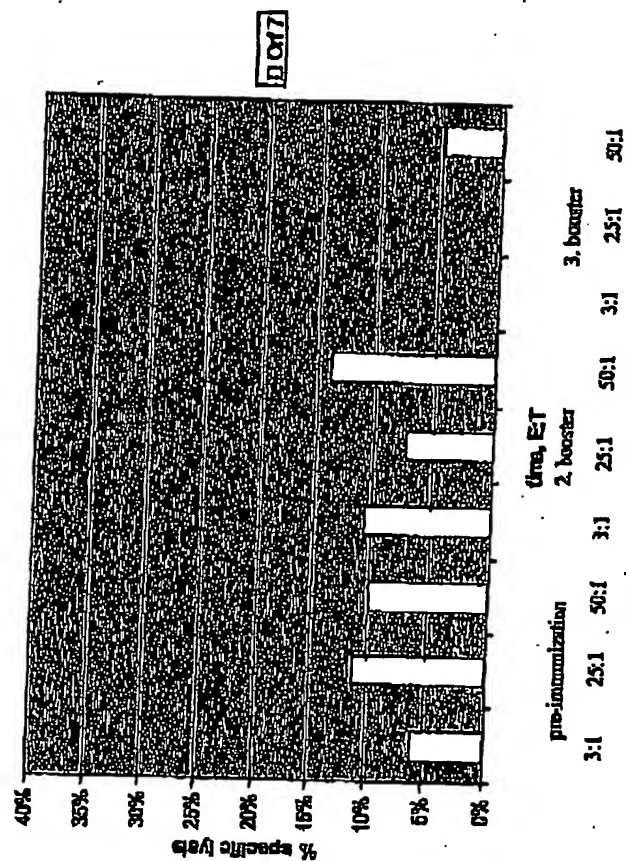
Fig. 15 a)



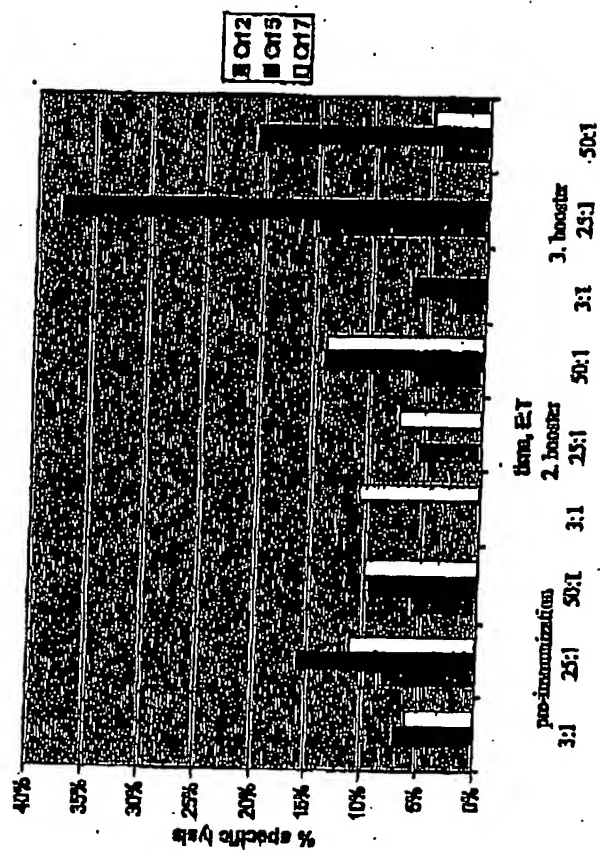
## Frieda, specific lysis Orf 2



## Frieda, specific lysis Orf 7



## Frieda, specific lysis



## Frieda, specific lysis Orf 5

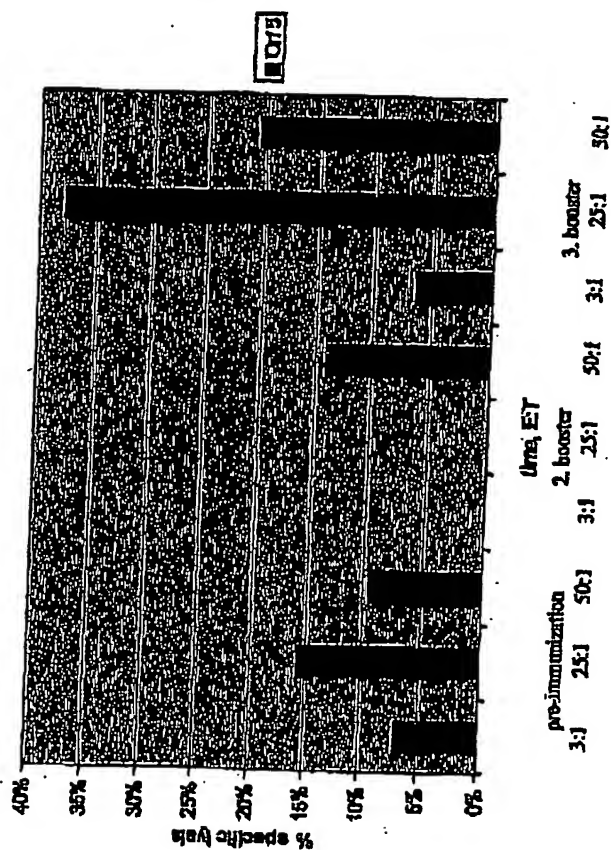
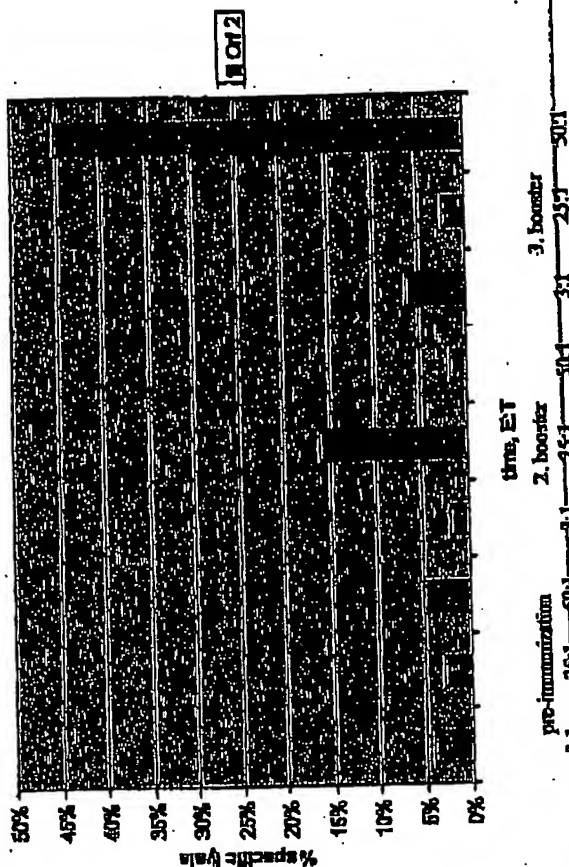
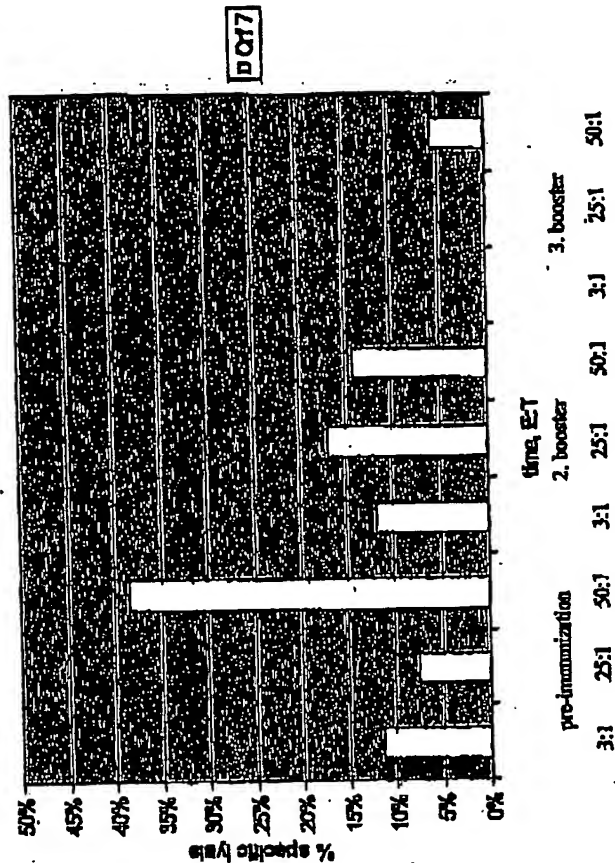


Fig. 15 b)

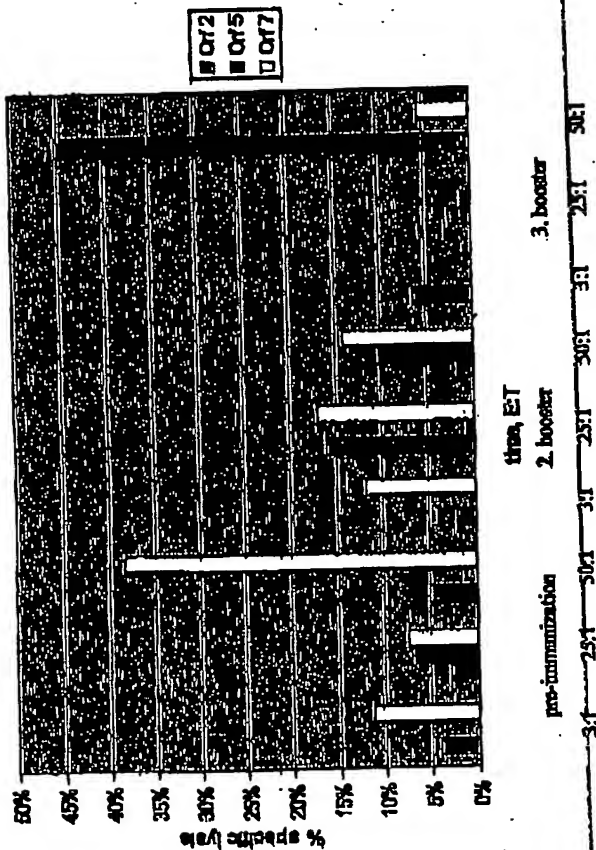
Friedrich, specific lysis Orf 2



Friedrich, specific lysis Orf 7



Friedrich, specific lysis



Friedrich, specific lysis Orf 5

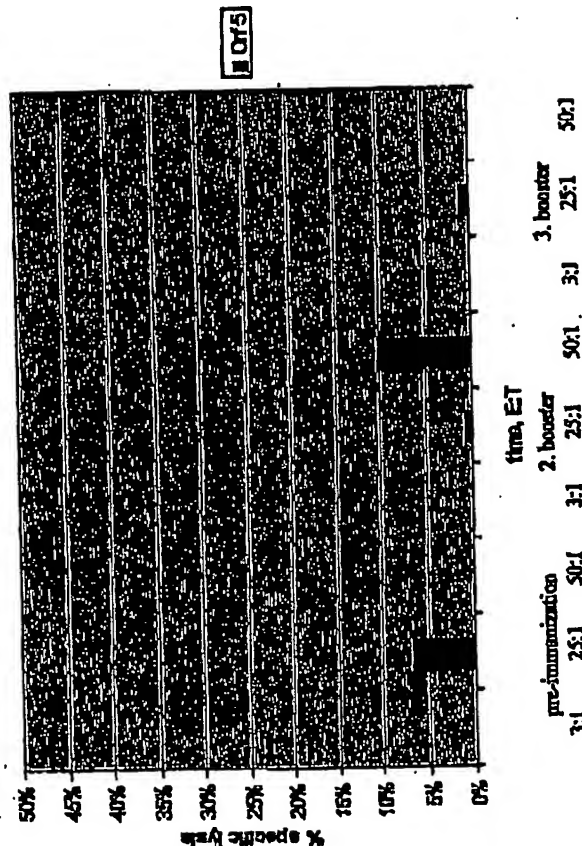
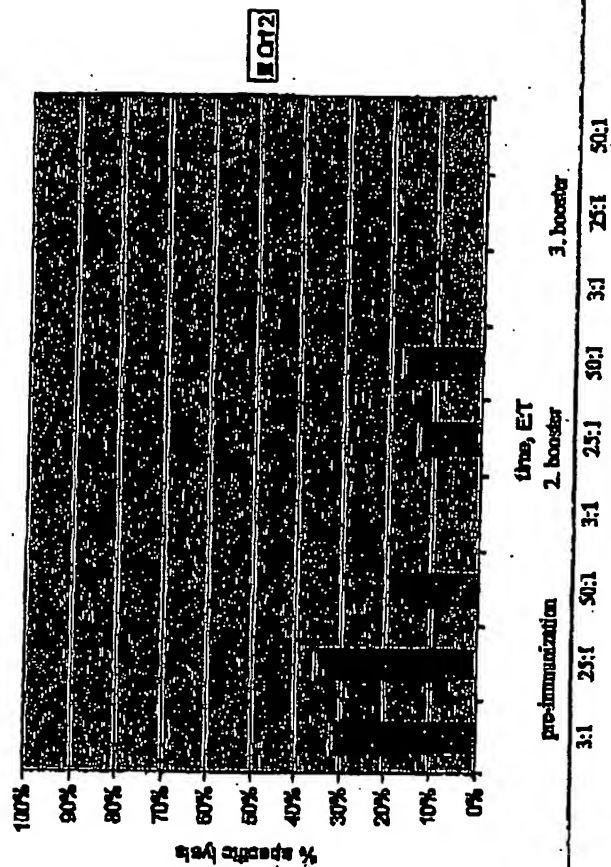
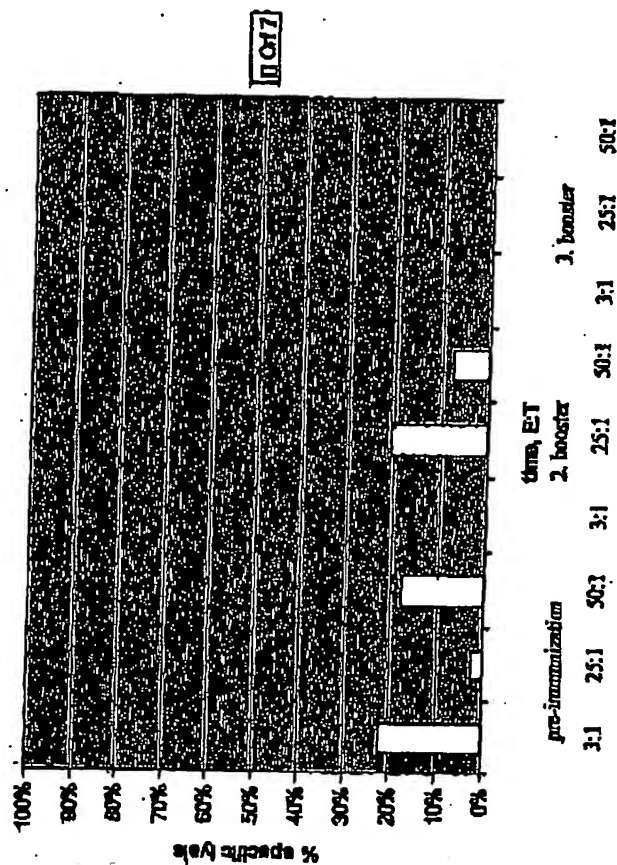


Fig. 15 c)

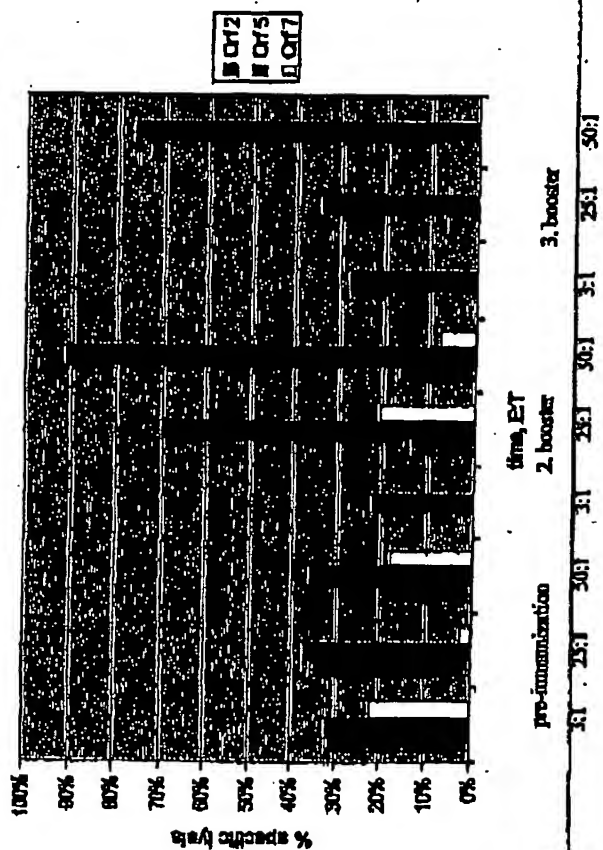
Jessy, specific lysis Orf 2



Jessy, specific lysis Orf 7



Jessy, specific lysis



Jessy, specific lysis Orf 5

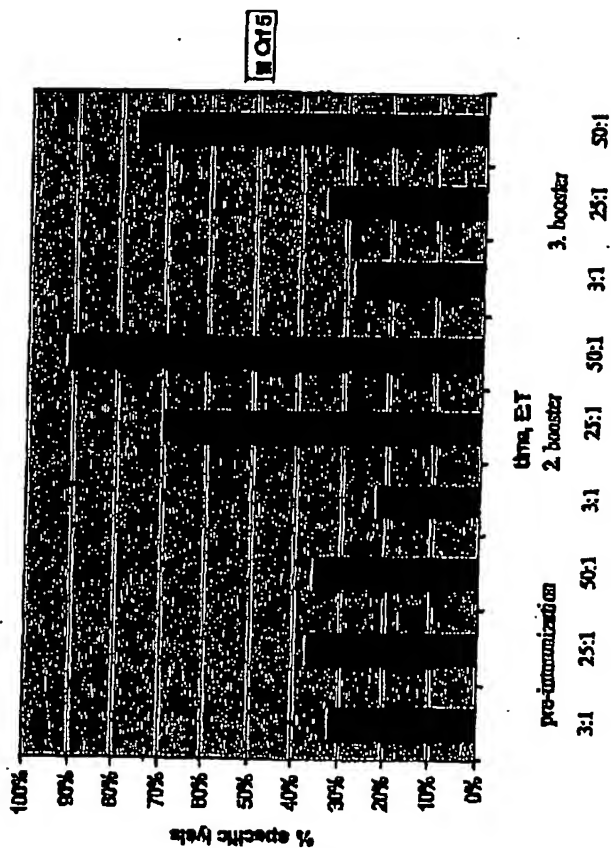
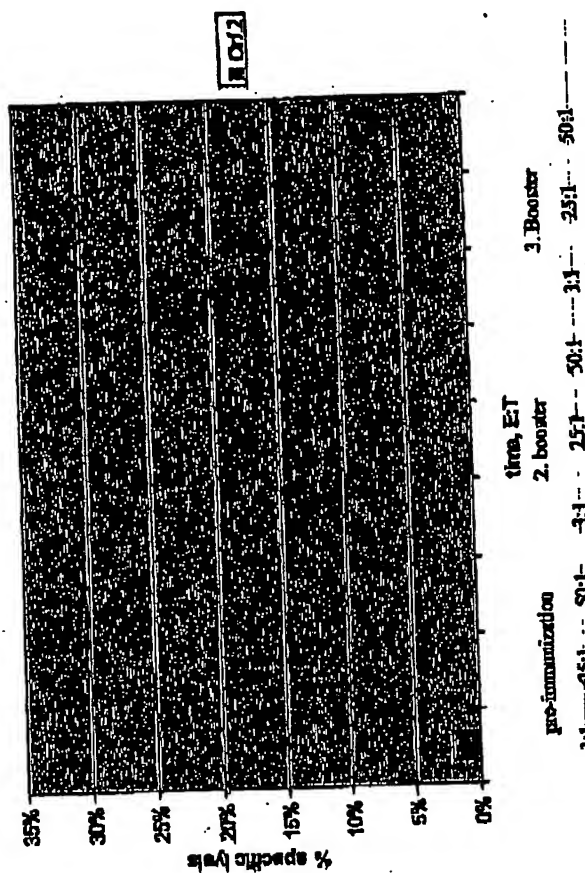


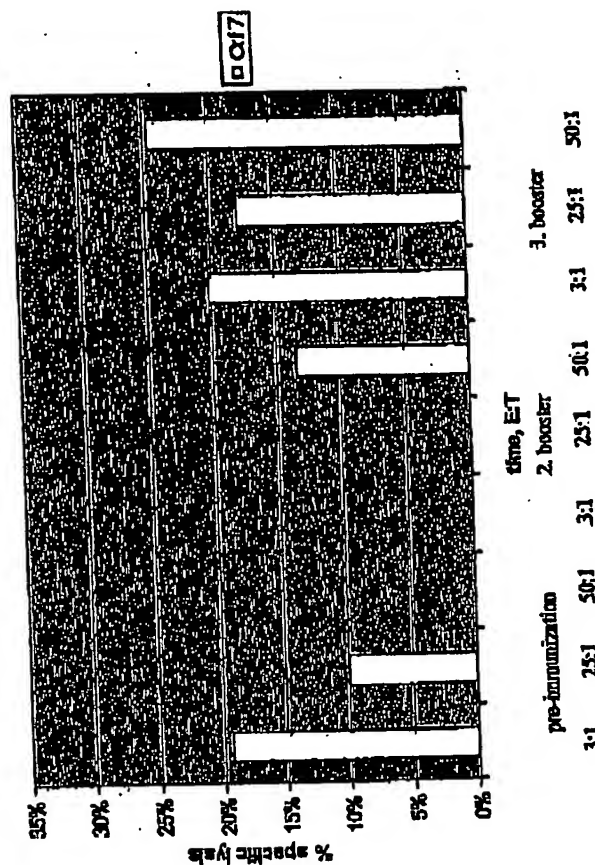
Fig. 15 d)

20

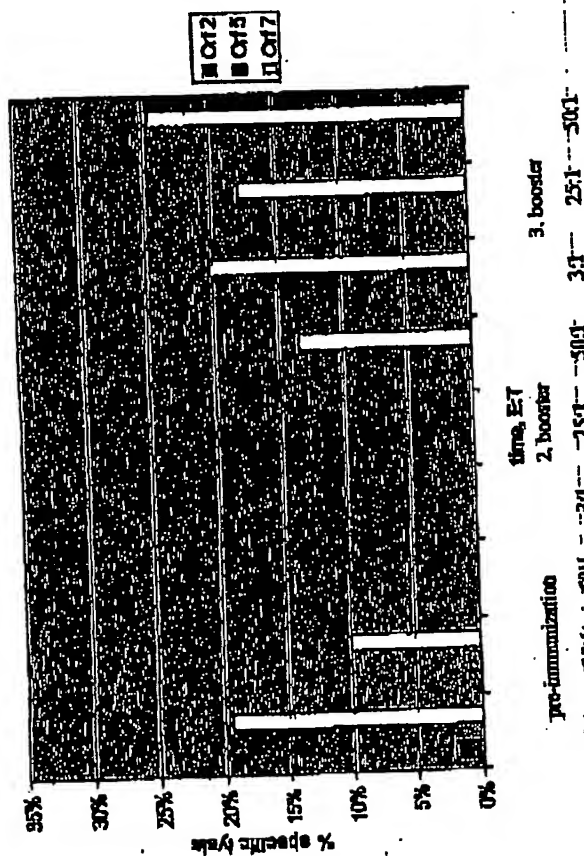
b, specific lysis Orf 2



Neke, specific lysis Orf 7



Neke, specific lysis



Neke, specific lysis Orf 5

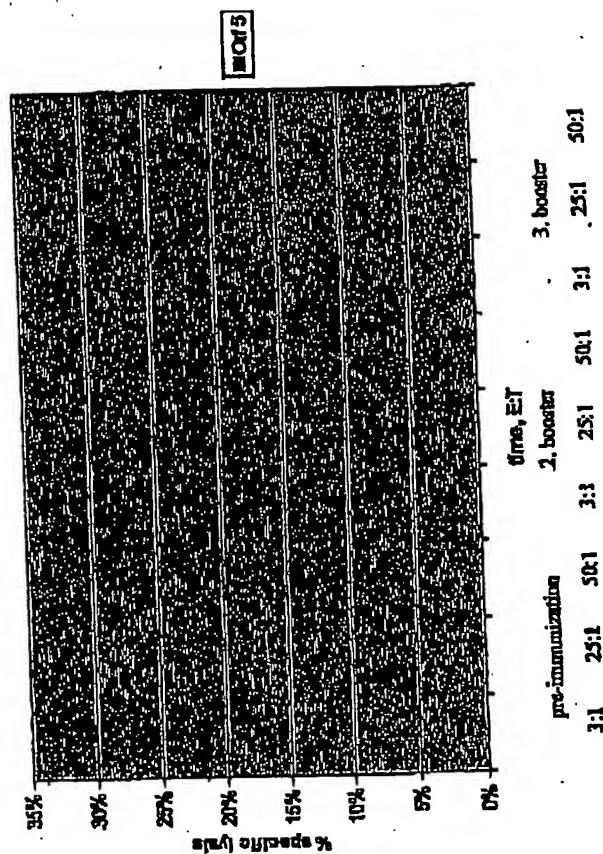


Fig. 15 e)

**This Page is Inserted by IFW Indexing and Scanning  
Operations and is not part of the Official Record**

**BEST AVAILABLE IMAGES**

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☒ **BLACK BORDERS**
- ☐ **IMAGE CUT OFF AT TOP, BOTTOM OR SIDES**
- ☐ **FADED TEXT OR DRAWING**
- ☒ **BLURRED OR ILLEGIBLE TEXT OR DRAWING**
- ☐ **SKEWED/SLANTED IMAGES**
- ☐ **COLOR OR BLACK AND WHITE PHOTOGRAPHS**
- ☐ **GRAY SCALE DOCUMENTS**
- ☐ **LINES OR MARKS ON ORIGINAL DOCUMENT**
- ☐ **REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY**
- ☐ **OTHER:** \_\_\_\_\_

**IMAGES ARE BEST AVAILABLE COPY.**

**As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.**

**THIS PAGE BLANK (USPTO)**